Inhibition of phorbol ester stimulated superoxide production by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG); fact or artefact?

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OAG-stimulated superoxide (O_2^-) production by HL-60 granulocytes showed enantiomeric specificity but reached a maximum of only 5% of that produced by either phorbol myristate acetate (PMA) or phorbol dibutyrate (PDBu). At 10–100 μM, OAG displaced specifically-bound [^{3}H]PDBu from intact HL-60 cells by only 25%, suggesting limited cell penetration. OAG (10–100 μM) also inhibited PDBu-stimulated O_2^- production by 25%; this inhibition was enantiomerically specific. However, at a lower concentration (3 μM), both enantiomers of OAG fully blocked O_2^- production stimulated by PMA (0.5 μM). This inhibition is probably artefactual, due to the hydrophobic PMA physically associating with OAG in the extracellular fluid.

Protein kinase C *Phorbol ester* *Diacylglycerol* *(HL-60 cell)* *Superoxide*

1. INTRODUCTION

The phospholipid- and calcium-dependent protein kinase C (PKC) is now recognised as playing a key role in the transduction of signals generated by many agonist-receptor interactions [1], being activated by diacylglycerol generated in the plasma membrane from phosphatidylinositol-4,5 bisphosphate breakdown [2]. In addition phorbol esters, which bear structural relationships to diacylglycerols, also bind to and activate the kinase [3]. Activation of PKC has been implicated in many diverse physiological responses including stimulation of degranulation and the oxidative burst by polymorphonuclear leukocytes [4,5].

We have used a model neutrophil system, dimethyl sulphoxide-differentiated HL-60 cells, to study activation of PKC by phorbol esters and diacylglycerols [6,7]. The generation of superoxide (O_2^-) by HL-60 granulocytes is stimulated dramatically by phorbol esters [6]. Unlike a number of other responses, such as degranulation, O_2^- production appears to be independent of changes in cytosolic calcium [8]. Generation of O_2^- thus provides a convenient and direct functional measurement of PKC activation.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

HL-60 promyelocytes were grown as suspension cultures in RPMI-1640 medium (Gibco), supplemented with 15% (v/v) heat-inactivated foetal calf serum (low endotoxin, Gibco) and a further 2 mM L-glutamine (Flow Laboratories). Cells were seeded at a density of 0.25×10^6/ml and subcultured twice weekly by resuspending in fresh medium so that their density did not exceed 2×10^6/ml. Cells were induced to differentiate along recognisable myeloid lines by the addition of dimethyl sulphoxide to a final concentration of 1.3% (v/v) and were used between 6 and 9 days later.

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2.2. *Superoxide measurement*

Superoxide anion production by the NADPH-dependent oxidase was measured as the reduction of ferricytochrome-*c* by a modification of the method of Cohen and Chovaniec [9]. HL-60 granulocytes were washed twice and resuspended at 0.3×10^6/ml in Hanks' balanced salt solution buffered with 30 mM Hepes (pH 7.4) and gassed with O_2 before use. Cells (1 ml) were added to plastic cuvettes containing 1 ml of 150 μM ferricytochrome-*c* (Sigma, Horse Heart type VI) dissolved in Hanks' solution. Samples were warmed for 5 min at 37°C before being placed in a spectrophotometer (Pye Unicam SP1750) equipped with an automatic cell changer warmed to 37°C. Extinction at 550 nm was measured against air, changes being recorded continuously for either 15 or 20 min after adding the PKC activator. The amount of O_2^- produced was calculated from the change in absorbance; $\epsilon = 21000\ M^{-1} \cdot cm^{-1}$ (reduced minus oxidised forms). Changes in extinction could be abolished by the addition of superoxide dismutase (75 units, bovine erythrocyte, from Sigma) before cell activation. Diacylglycerols and phorbol esters were added in 2 μl of ethanolic solution to 2 ml of cell suspension.

2.3. *[^{3}H]Phorbol dibutyrate ([^{3}H]PDBu) binding*

Undifferentiated HL-60 cells were taken in the log phase of growth, washed twice and resuspended in Hepes-buffered Hanks' solution, as before. The binding assay was carried out in polypropylene tubes at 37°C, the assay mix (1 ml) containing 2.5×10^6 cells, 10 nM [^{3}H]PDBu (14–20 μCi/nmol) and diacylglycerol or unlabelled PDBu, as indicated in the text. The cell suspension was preincubated at 37°C for 12 min before additions were made, each being in 1 μl volume of ethanolic solution: 17 min later, 0.75 ml of the assay mixture was removed and filtered through a Whatman GF/C filter which was then washed 3 times with 4 ml of Hanks' solution at 4°C. Tritium associated with the filters was determined by dispersing them in 5 ml scintillant (Picofluor) and counting in a Beckman scintillation counter. Non-specific binding is defined as that observed in the presence of 1000-fold excess of unlabelled PDBu.

2.4. *Isolation of protein kinase-C*

PKC was isolated from rat brain cytosol using the procedure of Niedel et al. [10] and its activity measured using the Triton X-100 mixed-micelle method described by Hannun et al. [11]. The enzyme preparation was characterised as containing PKC by virtue of histone-phosphorylating activity which was dependent on Ca^{2+} and phosphatidylserine and stimulated 8–15-fold by addition of 1,2-dihexanoyl-*sn*-glycerol (5 mol%), activation being specific for the 1,2-*sn*-enantiomer.

2.5. *Synthesis of diacylglycerols*

1-Oleoyl-2-acetyl-*sn*-glycerol was prepared essentially by the method of Mori et al. [12] starting from 1-oleoyl-*sn*-glycerol. The previously unreported 3-oleoyl-2-acetyl-*sn*-glycerol was prepared in an analogous manner starting from 3-oleoyl-*sn*-glycerol. Both products were shown to be free of 1,3-isomers by thin-layer chromatography and nuclear-magnetic resonance spectroscopy.

2.6. *Preparation of solutions*

Phorbol esters (Sigma) were stored as solutions (1 mg/ml) in acetone at −20°C. For use, the acetone was evaporated and the compound redissolved in ethanol (Fisons, Spectrograde) at 1000-times the required final concentration. Diacylglycerols were stored at −20°C until use when they were also dissolved in ethanol at 1000-times the final concentration.

3. RESULTS AND DISCUSSION

OAG stimulated O_2^- production by HL-60 granulocytes in an enantiomerically specific manner (fig.1). The response to the synthetic diacylglycerol was small when compared with a known activator of O_2^- production in HL-60 cells, the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (fMLP). However, combining the peptide with OAG produced a synergistic interaction which also displayed stereospecificity (fig.1). The phorbol esters PMA and PDBu are potent stimulators of O_2^- production in HL-60 granulocytes [6]. Fig.2 shows the dose-response curves for PMA, PDBu and OAG in these cells. The ED_{50} values for PMA and PDBu were 4.0×10^{-10} M and 6.3×10^{-8} M, respectively. PMA was

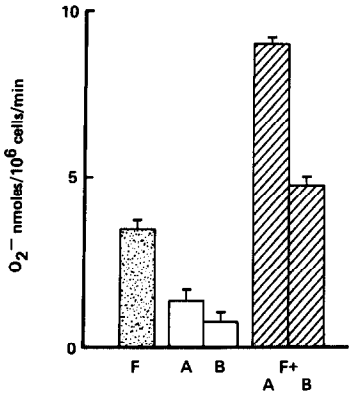

Fig.1. Superoxide production by HL-60 granulocytes. Synergistic interaction between OAG and the chemotactic peptide fMLP. F, FMLP (1 μM); A, 1-oleoyl-2-acetyl-*sn*-glycerol (100 μM); B, 3-oleoyl-2-acetyl-*sn*-glycerol (100 μM). Results are the means (± SE) of 4–11 individual determinations.

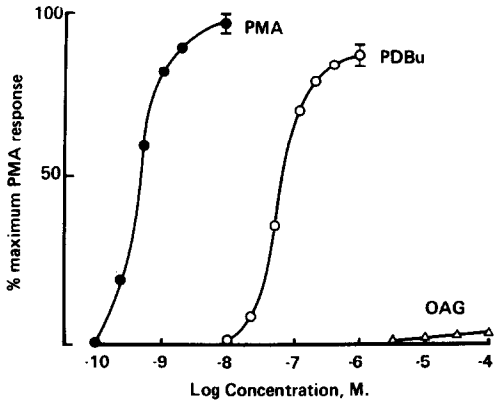

Fig.2. Diacylglycerol- and phorbol ester-stimulated O_2^- production by HL-60 granulocytes. Each point represents the mean (± SE) of 6 separate determinations. Maximum PMA response = 168 nmol $O_2^-/10^6$ cells in 20 min.

therefore approximately 100-times more potent than PDBu as a stimulus for O_2^- production; nevertheless both agents generated the same maximum response. In contrast, OAG produced only 5% of the maximum response elicited by the phorbol esters (fig.2).

Activation by OAG of partially purified PKC reached the same maximum as that induced by the phorbol esters (not shown). This contrasts sharply with the ability of OAG to stimulate O_2^- production (fig.2). Possible explanations are either that OAG is unable to penetrate to the sites on PKC that are accessible to phorbol esters in intact cells, or that the effective concentration of OAG is diminished through metabolism, as occurs in platelets [13]. The interpretation of restricted access was reinforced by the observation that OAG displaced specifically-bound [^{3}H]PDBu from intact HL-60 cells by a maximum of only 25% (fig.3).

An alternative explanation for these different maxima might have been that OAG is a partial agonist at the phorbol ester site on PKC. Although this clearly was not the case for the enzyme isolated from rat brain, it is conceivable that the PKC of HL-60 cells has an activation profile different from that of the brain enzyme. If OAG was a partial agonist at the phorbol ester site it should, by definition, competitively antagonise responses mediated by full agonists [14]. The action of OAG on either PDBu- or PMA-induced O_2^- production is shown in fig.4. OAG inhibited PDBu-stimulated O_2^- production by 25% and this effect was enantiomerically specific. The small degree of inhibition of the PDBu response by OAG is in agreement with the poor displacement of [^{3}H]PDBu from intact cells, suggesting perhaps that the inhibitory action of OAG is limited by its accessibility to the PDBu site on PKC.

In contrast to the PDBu response, PMA induced O_2^- production was completely inhibited by OAG at a concentration of 3 μM (fig.4). Furthermore, the inhibitory action of OAG was not enantiomerically specific. This lack of stereospecificity implies that the effect of OAG against PMA-

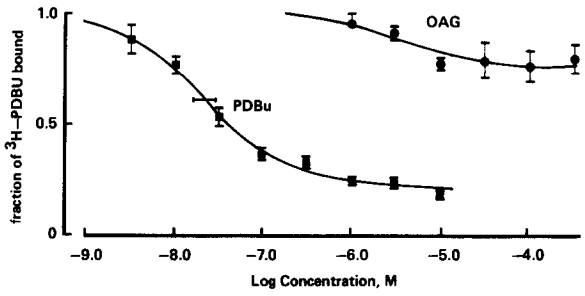

Fig.3. Displacement of [^{3}H]PDBu from HL-60 cells by PDBu and OAG. Each point represents the mean (± SE) of 9 separate observations.

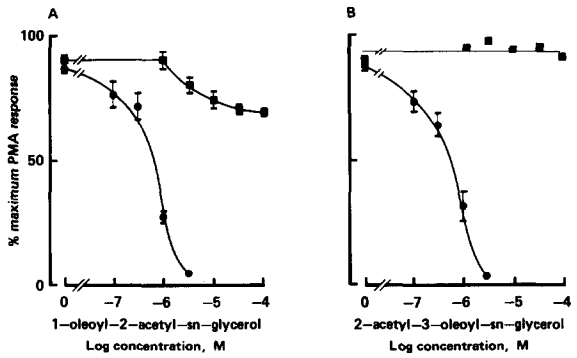

Fig.4. Inhibition of PMA (0.5 nM, ●)- and PDBu (100 nM, ■)-stimulated O_2^- production by OAG. (A) 1-Oleoyl-2-acetyl-*sn*-glycerol; (B) 2-acetyl-3-oleoyl-*sn*-glycerol. Each point represents the mean (± SE) of 3–5 separate experiments each in triplicate.

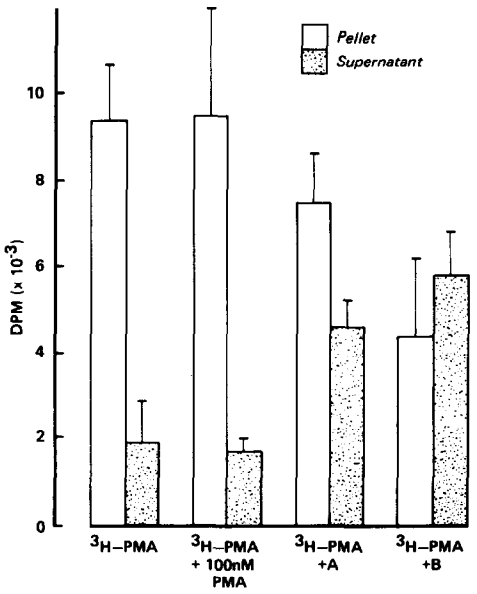

Fig.5. Effect of OAG on the distribution of [^{3}H]PMA between HL-60 cells and the supernatant. Each value represents the mean (± SD) of 4 separate determinations. (A) 1-Oleoyl-2-acetyl-*sn*-glycerol; (B) 3-oleoyl-2-acetyl-*sn*-glycerol.

induced responses is artefactual. The partition coefficients of PMA and PDBu differ by a factor of 10^4. Therefore, the extremely hydrophobic nature of PMA compared with PDBu may result in a physical association with OAG in the aqueous phase, particularly if the concentration of OAG is close to its critical micellar concentration. Consistent with this was the observation that OAG decreased the association of [^{3}H]PMA with the cell pellet and increased the amount of [^{3}H]PMA associated with the supernatant fraction (fig.5). As would be expected, this effect was not enantiomerically specific. Thus, a large part of the inhibitory action of OAG may be attributed to the ability of OAG to lower the concentration of PMA in HL-60 cells. This artefact would affect responses to PDBu to a lesser, possibly insignificant, degree because PDBu is much less hydrophobic than PMA.

From the above analysis, the inhibition by OAG of other PMA-induced responses, such as degranulation in human neutrophils (reported by O'Flaherty et al. [15]), can be explained as being artefactual. Clearly, studies which are designed to measure the inhibition of PKC-mediated responses must eliminate artefactual hydrophobic interactions, and we believe that the use of PDBu rather than PMA as an activator will reduce this possibility. Thus, the small but enantiomerically specific inhibition of PDBu-stimulated O_2^- production is probably not an artefact. The mechanism by which this inhibition occurs will be the subject of further investigation.

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