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Divergent effects of co-carcinogenic phorbol esters and a synthetic diacylglycerol on human neutrophil chemokinesis and granular enzyme secretion

[1]Sussan Nourshargh & [2]J.R.S. Hoult

Department of Pharmacology, King's College (KQC), Strand, London WC2R 2LS

1 The effects of two co-carcinogenic phorbol esters (phorbol myristate acetate (PMA) and phorbol dibutyrate (PDBu)) and a synthetic diacylglycerol (OAG, 1-oleoyl-2-acetyl-glycerol), which all stimulate protein kinase C, were compared with two inactive phorbol compounds (4α-phorbol and 4α-phorbol didecanoate (4α-PDD)) on three functional properties of stimulated human polymorphonuclear leukocytes (PMNs): release of granular enzymes lysozyme and β-glucuronidase, chemokinesis, and changes in cytoplasmic free calcium $[Ca^{2+}]_i$.

2 PMA, PDBu and the diacylglycerol, OAG, all caused a dose-dependent and slow (max by 15 min) release of small amounts of lysozyme with much less β-glucuronidase and no release of cytoplasmic lactate dehydrogenase. Release was unaffected by removal of extracellular Ca^{2+}.

3 PMA, PDBu and OAG inhibited random movement of the cells, did not cause chemokinesis and induced a slow reduction in the basal $[Ca^{2+}]_i$, as measured by the quin-2 method.

4 PMA, PDBu and OAG increased the capacity of five independently-acting stimulants (N-formyl-Met-Leu-Phe, leukotriene B_4, C5a des-Arg, platelet activating factor and A23187) to cause release of lysozyme and β-glucuronidase but strongly inhibited PMN chemokinesis induced by the same five agents and reduced the stimulant-induced increases in $[Ca^{2+}]_i$.

5 PMA was always more potent than PDBu and much more potent than OAG in eliciting these stimulatory or inhibitory effects on human PMNs. In all tests, 4α-phorbol and 4α-PDD were inactive.

6 The results confirm that stimulation of the diacylglycerol/protein kinase C system in human PMN, either by active phorbol esters or the synthetic diacylglycerol, causes bidirectional effects on human PMN function. In particular, activation of the C-kinase causes inhibition of stimulated neutrophil motility, whereas the secretory functions of the cells are enhanced.

Introduction

Recently we found that although the secretion of lysozyme and β-glucuronidase from human polymorphonuclear neutrophil leukocytes (PMNs) was enhanced by the co-carcinogenic phorbol myristate acetate (PMA), chemokinesis was strongly inhibited (Hoult & Nourshargh, 1985). In these experiments the neutrophils were stimulated with N-formyl-Met-Leu-Phe (FMLP). We concluded that these effects were mediated by the diacylglycerol/protein kinase C system (Nishizuka, 1984) because 4α-phorbol didecanoate (4α-PDD), which is not a co-carcinogen and which does not activate the C-kinase, neither inhibited chemokinesis nor enhanced granular enzyme secretion.

The purpose of the present study was to validate and extend these findings. Thus, we have compared the effects of two active tumour promoting phorbol esters which stimulate protein kinase C (PMA and phorbol dibutyrate, PDBu) with those of two closely related but inactive agents (4α-phorbol and 4α-PDD), and have also used a synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG) (Kaibuchi *et al.*, 1983; Kajikawa *et al.*, 1983). Additionally, we have used five different stimulants to activate human PMN chemokinesis and granular enzyme secretion. These are FMLP, leukotriene B_4 (LTB_4), C5a des-Arg, Paf (platelet activating factor, AGEPC) and A23187. Finally, we have also monitored the changes in

[1]Present address: Section of Vascular Biology, Clinical Research Centre, Harrow HA1 3UJ.
[2]Author for correspondence.

cytoplasmic free calcium $[Ca^{2+}]_i$ by use of the quin-2 technique.

Methods

Preparation of human PMN suspensions

Blood from healthy non-medicated adult donors was drawn by venepuncture into 0.1 vol 3.15% (w/v) trisodium citrate, and PMNs separated by standard procedures, allowing leukocytes and erythrocytes to sediment in 0.15 M NaCl containing 2% (w/v) dextran, followed by density gradient centrifugation on Hypaque/Ficoll and repeated cycles of hypotonic lysis to remove contaminating erythrocytes (see Nourshargh & Hoult, 1986, for full details). The PMN leukocytes were finally suspended in phosphate buffered saline (PBS, pH 7.4, composition mM: NaCl 138, Na_2HPO_4 8.1, KH_2PO_4 1.5, KCl 2.7 and glucose 0.1% w/v) at a concentration of 2×10^6 cells ml^{-1}. These suspensions contained more than 95% PMN and viability was established by trypan blue exclusion.

Incubation of PMN for enzyme release studies

Aliquots of PMN (2×10^6 cells ml^{-1}) were suspended in PBS containing $CaCl_2$ 0.6 mM and $MgCl_2$ 1 mM and preincubated at 37°C for 5 min with cytochalasin B 5 $\mu g\,ml^{-1}$ in 15 mm × 75 mm plastic tubes. After adding appropriate concentrations of stimulant agents or their vehicles (duplicate tubes) the cells were incubated with occasional shaking for a further 5 min (or longer, depending on the appropriate experimental protocol). The release reaction was terminated by centrifuging the tubes at 800 *g* for 20 min at 4°C, and the cell-free supernatants were either assayed immediately for release of lactate dehydrogenase (LDH, see below) or stored at −20°C prior to assay of β-glucuronidase and lysozyme.

LDH was assayed spectrophotometrically by using pyruvate as substrate in the presence of NADH (see Bergmeyer & Bernt, 1974). Lysozyme was assayed according to the method of Yuli *et al.* (1982). In this method aliquots of thawed PMN supernatants are incubated with suspensions of *Micrococcus lysodeikticus* and absorbance at 450 nm measured spectrophotometrically after quenching the reaction with ethanol. The enzyme β-glucuronidase was assayed spectrofluorimetrically (Barrett & Heath, 1979), using 4-methylumbelliferyl-β-D-glucuronide as substrate in pH 5.0 citrate buffer. In all cases, the enzyme release is expressed in terms of the total enzyme content of the cells, obtained by exposing the PMN to 0.2% (v/v) Triton X-100 for 5 min. All results are corrected for the spontaneous release of the enzymes from unstimulated cells carried through the same incubation procedure. In twelve consecutive experiments this was as follows: LDH < 7%, β-glucuronidase 4.3 ± 0.5% (range 2.0% to 7.4%), lysozyme 10.3 ± 1.2% (range 3.3% to 17.3%). Addition of stimulating agents was made using solutions diluted from stocks held in dimethylsulphoxide (DMSO); addition of similar dilutions of the vehicles alone did not cause significant levels of enzyme release from the PMNs.

Measurement of changes in cytoplasmic free calcium

Neutrophil suspensions (ca. 2×10^8 cells ml^{-1} in 10 mM HEPES-buffered PBS, pH 7.4, containing $CaCl_2$ 1 mM) were incubated for 15 min at 37°C with quin-2 ester 100 μM, then diluted ten fold and incubated for a further 45 min. After two washings by centrifugation, the cells were resuspended in the same buffer at 10^7 cells ml^{-1}, kept at room temperature and then monitored for changes in fluorescence after equilibration at 37°C in 1.5 ml volumes in a Perkin-Elmer model MPF-4 recording spectrophotofluorimeter with continuous stirring (excitation 339 nm, emission 492 nm). Levels of cytoplasmic free calcium were calculated according to the method of Tsien *et al.* (1982) with the modification suggested by Rink *et al.* (1983). Correction was also made for the leakage of quin-2 from the cells during the experimental period.

Chemokinesis assay

The agarose microdrop technique described by Smith & Walker (1980) was used. Liquified agarose gel (0.4% w/v in Eagle's minimum essential medium buffered with 20 mM HEPES) was added to an equal volume of cells (5×10^8 cells ml^{-1} in the medium) and 2 μl samples pipetted into each well of a 96-well microtitre plate. Samples of stimulating agents and pharmacological agents were added to each well in 100 μl of buffered medium (4 replicates per treatment) when the agarose droplets had solidified, and the plates were then incubated for 4 h at 37°C under 5% CO_2/95% O_2. The extent of migration of the circular leading front of the cells was measured after projecting the microscope image of the well on a TV monitor, using the average of > 4 separate radii. The percentage increase in migration of stimulated cells was calculated in relation to the extent of migration shown by unstimulated cells.

Materials

PMA (4β-phorbol-12-myristate-13-acetate, also known as TPA, 12-O-tetradecanoyl-phorbol-13-acetate), PDBu (4β-phorbol-12,13-dibutyrate), 4α-PDD (4α-phorbol-12,13-didecanoate), 4α-phorbol, OAG (1-oleoyl-2-acetyl-rac-glycerol), N-formyl-Met-

Leu-Phe (FMLP), Paf (1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine), cytochalasin B, 4-methyl-umbelliferyl-β-D-glucuronide, dried *Micrococcus lysodeikticus*, dextran, EGTA and sodium pyruvate were all purchased from Sigma Chemical Co Ltd (Poole). A23187 (Calimycin) was from Calbiochem (Bishops Stortford). Ficoll 400 and Isopaque 440 were from Pharmacia (Milton Keynes) and Vestric Ltd (Runcorn), respectively. C5a des-Arg purified from zymosan-activated rabbit plasma was a kind gift from Dr P. Jose, Clinical Research Centre, Harrow. Leukotriene B_4 was a kind gift from Dr J. Rokach (Merck Frosst Canada Inc, Quebec, Canada). Quin-2-tetra-acetoxy-methyl ester was from Lancaster Synthesis.

Results

Direct effects of phorbol esters and OAG as activators of human PMN

As shown in Figure 1, the co-carcinogens PMA and PDBu caused dose-dependent secretion of lysozyme and β-glucuronidase when exposed to the PMN for 15 min. 4α-Phorbol and 4α-PDD did not elicit enzyme secretion (Figure 1), even when added at 10^{-6} M for as long as 30 min. Phorbol ester-induced secretion was maximal within about 15 min (Figure 1b,d) and was greater for lysozyme than for β-glucuronidase. Indeed, lysozyme release was selective in that only at concentrations of PMA and PDBu of 10^{-7} M and above were amounts of secreted β-glucuronidase significantly greater than the spontaneous rates of release (see legend to Figure 1). PMA (ED_{50} approx. 1.5 nM) was more potent a secretagogue than PDBu (ED_{50} approx. 4 nM) and released larger amounts of both enzymes. None of the phorbol derivatives caused LDH release under these conditions (data not shown). The synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG), also caused dose-dependent secretion of small amounts of lysozyme and β-glucuronidase (Figure 1). Secretion was slow in onset, reaching maximal levels within 16–30 min (Figure 1f). Note that there was considerable variability of the data (such that, for example, none of the β-glucuronidase points in Figure 1e are significantly different from each other even though they show a clear dose-dependent trend).

Separate experiments (data not presented) showed that substitution of the 0.6 mM $CaCl_2$ solution with 10 mM EGTA did not affect the capacity of 10^{-10} M to 10^{-6} M PMA and 10^{-6} M to 5×10^{-4} M OAG to cause dose-dependent release of lysozyme from human PMN.

The effects of all four phorbol derivatives and OAG were tested on chemokinesis. None of these agents stimulated chemokinesis when added at concentrations ranging from 0.1 to 1000 nM (phorbol derivatives) or 0.1 to 100 μM (OAG). In fact, in some experiments PMA and PDBu reduced the basal PMN motility (e.g. in 4 out of 7 experiments PMA and PDBu caused 25.3 ± 5.8% and 17.6 ± 7.2% inhibition of migration, respectively).

Addition of PMA or PDBu at 10 to 100 nM to quin-2-loaded human PMN caused a slow and dose-dependent reduction in the basal fluorescence signal, corresponding to a reduction in cytoplasmic free calcium. 4α-Phorbol and 4α-PDD at concentrations up to 500 nM did not have this effect. In 4 out of 8 experiments, OAG at 0.1 mM also caused a similar fall in the basal fluorescence level of quin-2-loaded cells, but the reason for the variability was not established.

Effects of phorbol derivatives on PMNs stimulated by other agents

Preincubation of human PMN with low concentrations of active phorbol esters significantly increased the ability of a subsequently added agonist to cause secretion of β-glucuronidase and lysozyme. This is illustrated in Figure 2 for 10 nM PMA in relation to the lysozyme and β-glucuronidase log dose-secretion curves for LTB_4, and shows that the enhancement occurs throughout the dose-response curve. Figure 2 also shows that 4α-phorbol at 1 μM did not have any effect on agonist-induced secretion of the enzymes. Very similar results were obtained for enhancement of FMLP-induced secretion of lysozyme and β-glucuronidase by PMA but not by the inactive 4α-PDD and were shown in our earlier publication (Hoult & Nourshargh, 1985, Figure 1b and 1c).

As described in the Discussion, there is some controversy about the effects on PMN degranulation and enzyme secretion of co-treatment of the cells with degranulating stimulants and agents which activate protein kinase C. Thus we wished to establish the generality of the enhancing effects described above. Table 1 summarizes selected data from this set of experiments, so as to illustrate that the active phorbol esters (PMA and PDBu, both used at 10 nM) as well as OAG (used at 100 μM) but not 4α-PDD (1 μM) significantly augmented the secretion of both lysozyme and β-glucuronidase in response to all five stimulants tested (FMLP, LTB_4, C5a des-Arg, Paf and A23187). In all these experiments, the values for percentage release of enzymes by combinations of agents have been corrected for the (small) amount of enzyme release caused by the preincubating agent.

In marked contrast (Figure 3), the active phorbol esters potently and dose-dependently inhibited chemokinesis induced by all five stimulants (FMLP, Paf, C5a des-Arg, LTB_4 and A23187) with PMA more potent than PDBu on all four occasions when they were compared (Figure 3a to d). The IC_{50} values varied slightly depending on the nature of the chemokinetic

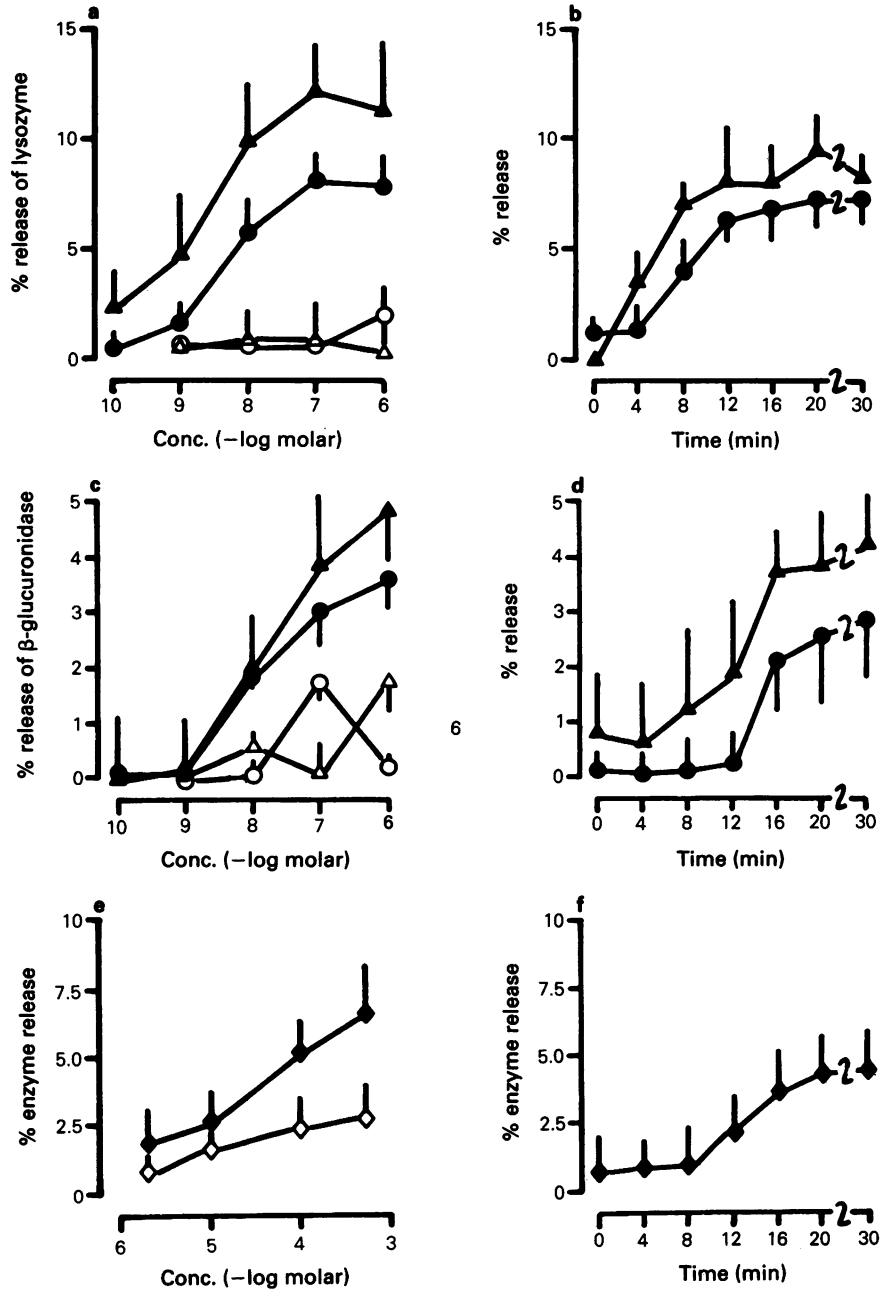

Figure 1 Effects of phorbol esters and a synthetic diacylglycerol on release of lysozyme and β-glucuronidase from cytochalasin B-treated human PMNs. Left hand panels (a) and (c) show dose-dependent effects of active phorbol esters (▲ = phorbol myristate acetate (PMA), ● = phorbol dibutyrate (PDBu)) compared to inactive phorbol esters (○ = 4α-phorbol, △ = 4α-phorbol didecanoate (4α-PDD)) on release of enzymes. The effects of 1-oleolyl-2-acetyl-glycerol (OAG) on release of lysozyme (◆) and β-glucuronidase (◇) are shown in (e). All incubations 15 min. The right hand panels (b), (d) and (f) (same symbols) show time course of enzyme release at fixed dose of stimulant (PMA 100 nM, ▲; PDBu 100 nM, ● and OAG 100 μM, ◆-lysozyme release). Note that (a) and (b) refer to lysozyme release and (c) and (d) are for β-glucuronidase. The basal rates of enzyme release (which were lysozyme 10.3 ± 1.2% and β-glucuronidase 4.3 ± 0.5%) have been subtracted from the values shown. Results show mean ± s.e.mean (bars) for at least 3 separate experiments, each test in duplicate.

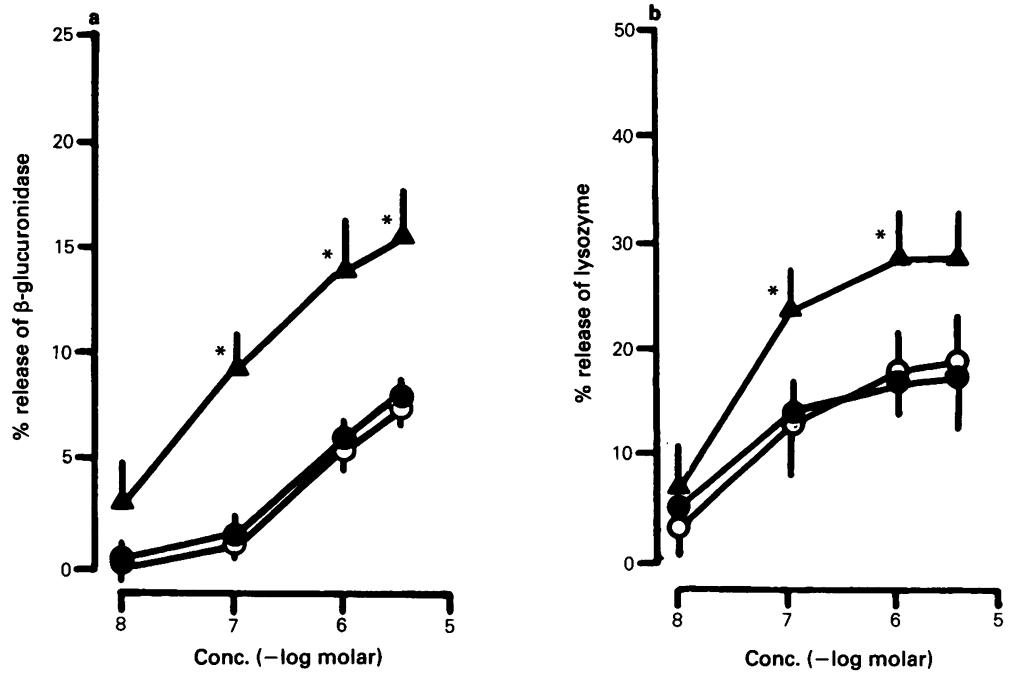

Figure 2 Enhancement of leukotriene B (LTB_4)-induced granular enzyme release from human PMNs by phorbol myristate acetate (PMA) but not by 4α-phorbol. Cells were preincubated for 5 min with vehicle (○), 10 nM PMA (▲) or 1 μM 4α-phorbol (●) before addition of various concentrations of LTB_4 and further incubation for 5 min: (a) shows β-glucuronidase, (b) shows lysozyme. Results show mean ± s.e.mean (bars) from 3 or 4 separate experiments (each test in duplicate), and results for PMA have been corrected for the small amount of release induced by this agent alone (cf. Figure 1). Significant difference from control, $*P < 0.05$, using Student's unpaired t test.

Table 1 Enhancement of agonist-induced secretion of lysozyme and β-glucuronidase from human PMN by active co-carcinogenic phorbol esters and OAG

	% enzyme release				
	Control	*PMA*	*PDBu*	*4α-PDD*	*OAG*
FMLP	13.3 ± 1.9	19.6 ± 1.1*	18.3 ± 0.5*	12.7 ± 1.6	18.1 ± 0.4*
	23.4 ± 2.1	27.6 ± 2.8	29.5 ± 2.3*	24.7 ± 3.3	26.4 ± 2.3
LTB_4	7.5 ± 1.1	15.8 ± 1.9**	12.9 ± 1.6*	7.1 ± 0.9	13.3 ± 1.3*
	12.6 ± 3.3	19.3 ± 2.8*	17.4 ± 2.9	14.3 ± 1.7	16.5 ± 1.3
C5a des-Arg	10.1 ± 2.1	18.6 ± 0.8**	16.4 ± 0.8*	9.3 ± 1.3	15.9 ± 1.1*
	17.4 ± 2.1	24.8 ± 3.3*	22.5 ± 1.3*	15.3 ± 3.1	19.5 ± 2.1
Paf	15.1 ± 1.5	23.3 ± 2.0*	19.4 ± 0.6*	14.5 ± 1.1	21.5 ± 1.4*
	20.6 ± 1.8	25.3 ± 2.2*	26.1 ± 1.2**	22.3 ± 2.3	24.6 ± 1.2*
A23187	21.1 ± 1.3	27.3 ± 0.9**	26.8 ± 1.1*	20.9 ± 0.6	25.3 ± 1.0*
	35.9 ± 2.3	43.7 ± 2.8**	42.6 ± 2.5*	37.9 ± 1.9	40.3 ± 2.1
PMA	5.3 ± 1.8	6.6 ± 2.1	4.9 ± 1.3	5.1 ± 1.6	4.0 ± 0.8
	10.3 ± 2.5	13.6 ± 2.4	12.9 ± 3.7	7.6 ± 2.9	8.1 ± 1.7

Results show mean ± s.e.mean from 4 separate experiments. Cells were preincubated with phorbol esters or OAG for 5 min (phorbol myristate acetate (PMA) and phorbol dibutyrate (PDBu) = 0.01 μM, 4α-phorbol didecanoate (4α-PDD) = 1 μM, 1-oleolyl-2-acetyl-glycerol (OAG) = 100 μM) then treated with stimulants for 15 min (N-formyl-Met-Leu-Phe (FMLP), platelet activating factor (Paf) and A23187 = 1 μM, leukotriene B_4 (LTB_4) and C5a des-Arg = 0.3 μM and PMA = 0.1 μM). The values have been corrected for the release of enzymes induced by the preincubating agents on their own, where relevant. For each stimulant, the top line gives β-glucuronidase release and the lower line gives lysozyme. The significance of differences compared to control cells without preincubation are $*P < 0.05$ and $**P < 0.01$ (Student's unpaired t test).

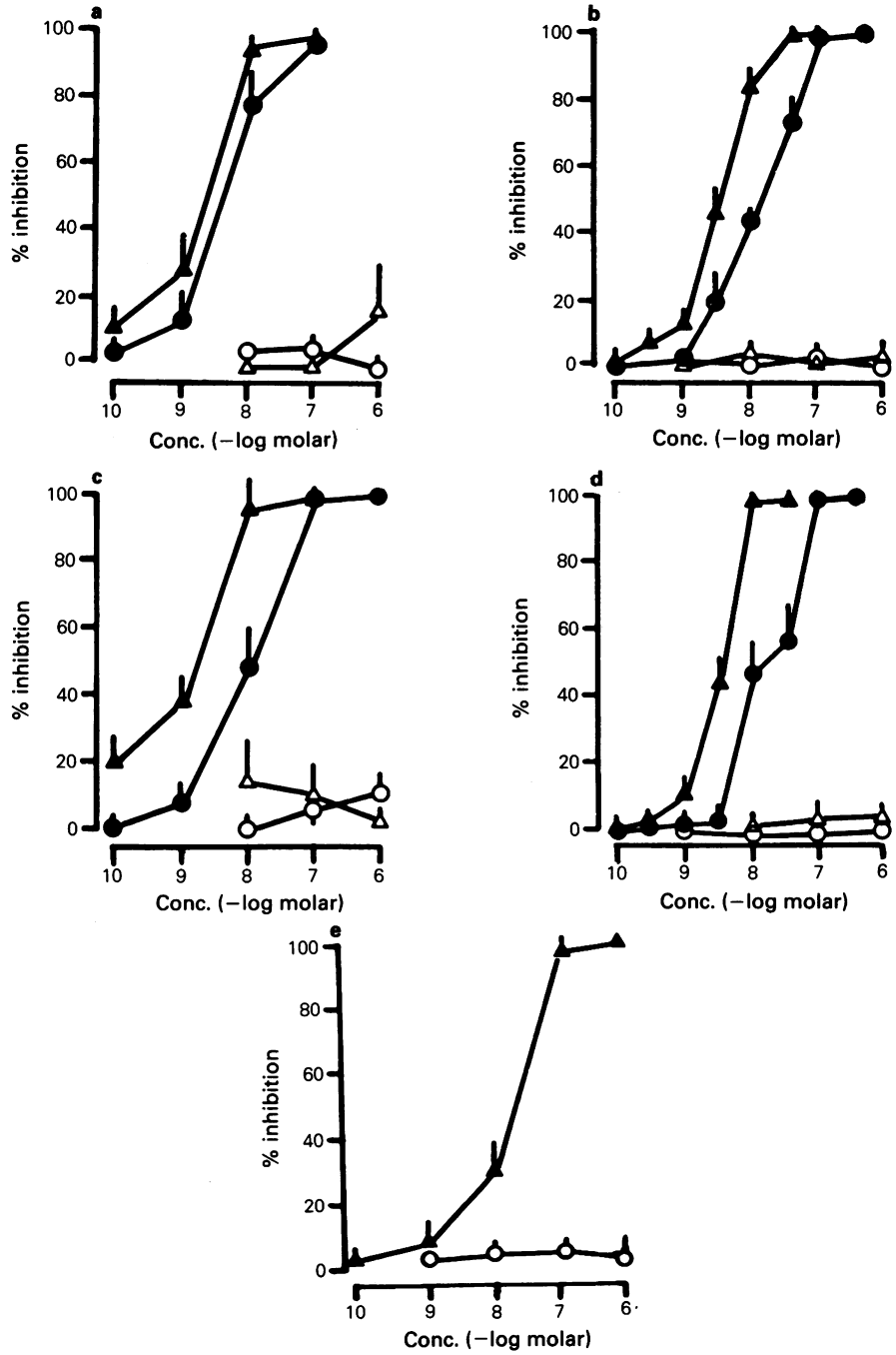

Figure 3 Dose-dependent inhibition of stimulated chemokinesis of human PMN by phorbol myristate acetate (PMA) and phorbol dibutyrate (PDBu) but not by inactive phorbol esters. Cells were stimulated by 10 nM C5a des-Arg (a), 1 nM N-formyl-Met-Leu-Phe (FMLP) (b), 10 nM platelet activating factor (Paf) (c), 1 nM leukotriene B_4 (LTB_4) (d) or 100 nM A23187 (e) and treated simultaneously with various concentrations of PMA (▲), PDBu (●), 4α-phorbol didecanoate (4α-PDD) (△) or 4α-phorbol (○). Extent of chemokinesis was measured in terms of the distance travelled by the leading front of the cells after 4 h incubation at 37°C. Results show mean ± s.e.mean (bars) from 3 experiments (except A23187, one experiment), each dose point assayed in quadruplicate. Data for FMLP with PMA and 4α-PDD were shown in Hoult & Nourshargh, 1985 (Figure 1d).

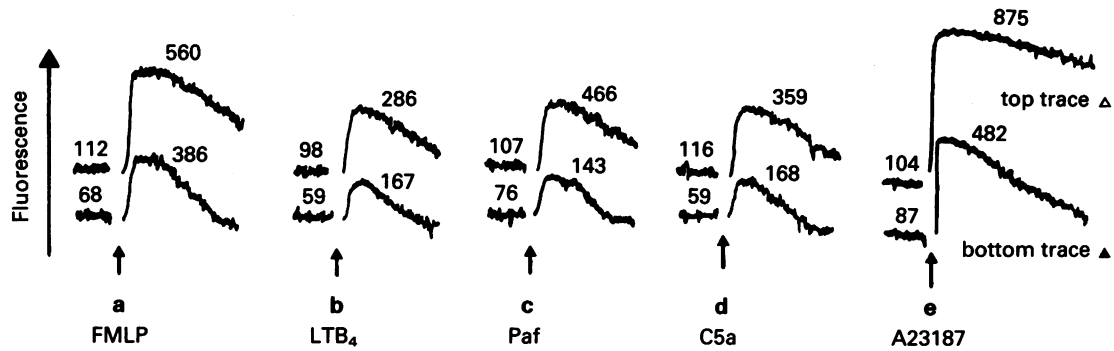

Figure 4 Inhibitory effects of phorbol myristate acetate (PMA) on stimulated increases in cytoplasmic free calcium in quin-2-loaded human PMNs. The cells were preincubated for 5 min with 10 nM PMA (▲) or its vehicle (△), then stimulated (at the arrow) with 100 nM concentrations of N-formyl-Met-Leu-Phe (FMLP) (a), leukotriene B_4 (LTB_4) (b), platelet activating factor (Paf) (c), C5a des-Arg (d) or A23187 (e). The numbers on the traces are the calculated concentrations in nM of $[Ca^{2+}]_i$.

Results are representative of at least 3 similar observations. Note that PMA not only reduces the maximal level of free cytoplasmic calcium induced by the stimulant, but also accelerates its rate of return towards the pre-stimulation value.

stimulant, ranging from 1.6 to 3.7 nM for PMA and 4.1 to 16.0 nM for PDBu. Against A23187, the IC_{50} for PMA was approximately 20 nM (Figure 3e). Both 4α-phorbol and 4α-PDD failed to influence chemokinesis induced by any of the five stimulants (Figure 3). OAG at 100 μM also inhibited chemokinesis induced by FMLP, LTB_4, Paf acether and C5a des-Arg (used at the concentrations shown in Figure 3) by 33.6 ± 5.4%, 24.3 ± 7.2%, 40.3 ± 10.3% and 55.9 ± 7.8%, respectively ($n = 4$), but as the s.e.mean values show, the reproducibility of the effects was not good. Higher concentrations of OAG could not be tested to establish full dose-inhibition curves on account of the inhibitory effects of the vehicle. In all these experiments, the stimulant agent and the phorbol derivatives (or OAG) were added simultaneously at the start of the 4 h chemokinesis period.

The effects of the phorbol esters on stimulant-induced increases in cytoplasmic free calcium were investigated. Figure 4 shows that regardless of the nature of the stimulant (FMLP, Paf, C5a des-Arg, LTB_4 and A23187), preincubation for 5 min with PMA considerably reduced the magnitude of the stimulant-induced increase in cytoplasmic free calcium and increased the rate at which the increased level returned towards the pre-stimulation value. Note also in Figure 4 that the basal values for cytoplasmic free calcium were lower in the PMA-pretreated samples than in vehicle-treated controls, reflecting its effects on basal calcium levels as described in the previous section. These effects of PMA were found to be dose- and time-dependent (greater with longer preincubation) and were shared by PDBu but not by 4α-PDD (data not shown). 4α-Phorbol and OAG were not tested in these experiments.

Discussion

Our aim was to extend our previous finding (Hoult & Nourshargh, 1985) that PMA, whilst enhancing secretion from the azurophil and specific granules, inhibits chemokinesis and the increases in cytoplasmic free calcium. The divergent inhibitory and stimulatory effects of PMA were attributed to its recognised ability to activate protein kinase C (Nishizuka, 1984). The present results do indeed support this hypothesis. Thus PMA and a second active phorbol ester, PDBu, have the same effects on degranulation, changes in cytoplasmic calcium and chemokinesis (chemically-stimulated random locomotion) induced by four other neutrophil stimulants (LTB_4, Paf, C5a des-Arg and A23187), whereas the two inactive phorbol compounds do not have this profile. Our results also indicate that stimulation of protein kinase C in human neutrophils results in cellular changes distal to receptor occupancy (FMLP, LTB_4, Paf, C5a des-Arg) or calcium entry (A23187) at sites which are common to the ability of these agents to induce degranulation or chemokinesis. The common target in the degranulation pathway may of course be different from that involved in the motility response.

That these pleiotropic responses to the phorbol compounds are due to the stimulation of the calcium-activated phospholipid-dependent protein kinase C is supported by the facts that (a) the order of potency with PMA more potent than PDBu, with 4α-phorbol and 4α-PDD inactive, was shown for all responses (Figures 1 and 3, Table 1 and text), and is the same as for activation of protein kinase C (Castagna *et al.*, 1982), binding to phorbol ester receptors (Goodwin & Weinberg, 1982; Niedel *et al.*, 1983) and biological

potencies (Goodwin & Weinberg, 1982; Blumberg *et al.*, 1984); and (b) OAG had the same spectrum of action. This synthetic diacylglycerol intercalates into the membrane, thereby mimicking diacylglycerol, the endogenous substrate for protein kinase C (Kaibuchi *et al.*, 1983). However, it should be noted that diacylglycerol is formed transiently as a result of receptor-activated phosphoinositide turnover (Michell *et al.*, 1981; Cockroft, 1982; Nishizuka, 1984; Berridge & Irvine, 1984), whereas phorbol esters and OAG may not be rapidly removed, leading to a prolonged and unphysiological stimulation of the kinase.

Our data showing a rather selective but slow release of lysozyme (used in these experiments as a marker for exocytosis from the 'specific' or 'secondary' granules) by PMA, PDBu and OAG (Figure 1) extend previous similar observations made for PMA in human and rabbit neutrophils (e.g. Estensen *et al.*, 1974; Schell-Frederick, 1974; Wright *et al.*, 1977; Gallin *et al.*, 1978; Lehmeyer *et al.*, 1979; Sha'afi *et al.*, 1983) and for OAG in human neutrophils (O'Flaherty *et al.*, 1984). Exocytosis of specific granules occurs without mobilization of cytoplasmic calcium (Sha'afi *et al.*, 1983), and is independent of extracellular calcium (Goldstein *et al.*, 1974; these results). It is also unaffected by pretreatment with cytochalasin B (Lehmeyer *et al.*, 1979), unlike degranulation responses induced by receptor-acting ligands.

Therefore a protein kinase C-dependent step appears sufficient to activate the exocytotic mechanism for specific but not azurophil granules, suggesting a biochemical difference in the way in which these organelles function. This is also supported by the studies of Gomperts *et al.* (1986) and by previous experiments showing that treatment of human neutrophils with either $CaCl_2$ (in divalent cation-free buffer) or low concentrations of A23187, both of which cause substantial elevations of $[Ca^{2+}]_i$, elicited only release of specific granules (Goldstein *et al.*, 1974; Wright *et al.*, 1977; Nourshargh, 1986). Similarly, saponin-treated permeabilized human neutrophils exposed to $CaCl_2$ release lysozyme but not β-glucuronidase (Smolen & Stoehr, 1985). Thus, in summary, exocytosis of the specific granules of neutrophils can be initiated either by direct activation of protein kinase C or by elevation of $[Ca^{2+}]_i$, but in both cases is slow in onset and quantitatively much less than when caused by activation of the cells by chemotactic stimulants acting on surface receptors.

This may be explained by assuming that optimal degranulation responses depend on the synergistic interactions between stimulation of protein kinase C and increased levels of cytoplasmic free calcium, according to the model developed by Nishizuka's group and widely discussed in recent years (e.g. Nishizuka, 1984; 1986; Berridge & Irvine, 1984; Drummond & MacIntyre, 1985). Recently, Dougherty & Niedel (1986) have shown that agents which elevate cytosolic Ca^{2+} (A23187 and FMLP) upregulate the binding of PDBu to its receptor in differentiated HL-60 cells, and have suggested that this may be the biochemical mechanism in leukocytes for the synergism between activators of the calcium and protein kinase C pathways.

The same arguments might explain why PMA, PDBu and OAG (but not 4α-phorbol or 4α-PDD) enhance the capacity of FMLP, LTB_4, Paf-acether, C5a des-Arg and A23187 to cause degranulation (Figure 2, Table 1). Although all five stimulants themselves certainly cause mobilization of cytoplasmic free calcium (Figure 4) and may promote transient diacylglycerol production (see below), additional stimulation of the kinase might be expected to enhance the physiological response, even though the kinase causes a reduction in the apparent mobilization of calcium. Similar results for enhanced degranulation have been found by Goldstein *et al.* (1975) (PMA and serum-treated zymosan, release of β-glucuronidase) and O'Flaherty *et al.* (1984) (diacylglycerols with various stimulants, enhanced release of both lysozyme and β-glucuronidase), but they contrast sharply with those of Naccache *et al.* (1985). Using elicited rabbit peritoneal neutrophils (i.e. cells subjected to chemotactic stimulation before experimentation), Naccache *et al.* showed that FMLP- and LTB_4-induced release of azurophil and specific granules was inhibited up to 80% by PMA at 160 nM (threshold for inhibition approx. 10 nM, the concentration used in our experiments, see Figure 3). Inhibition was evident within 1 min and maximal by 3 min preincubation, times sufficient for protein phosphorylation to have occurred (cf. our experiments: 5 min preincubation before inducing degranulation), but was not caused by 4α-PDD. The authors concluded (Naccache *et al.*, 1985) that the inhibition of agonist-induced degranulation was due to the ability of the phorbol ester to antagonize calcium mobilization by acting as a receptor uncoupling agent (agonist-induced but not A23187-induced increases in cytosolic $[Ca^{2+}]_i$ were also markedly inhibited, although not quantitated), perhaps by an interaction with the GTP-regulatory site. Guanine nucleotide regulatory proteins are implicated in receptor-mediated neutrophil responses, but not in those initiated by A23187 or PMA (see Verghese *et al.*, 1986). This explanation of Naccache *et al.* appears unlikely to apply to the present experiments on human neutrophils: our results (Figure 4) show that elevation of cytoplasmic Ca^{2+} caused by A23187 is also blunted by PMA in the same way as that due to receptor-acting agonists, a result in agreement with others (see below).

These differences between rabbit and human neutrophils are very puzzling. However, there may be

fundamental differences between the two species in the transduction pathways for agonist-induced degranulation. Alternatively, the difference may relate to the altered physiological behaviour of elicited versus peripheral neutrophils (cf. differences in their ability to aggregate, Bray *et al.*, 1980).

That stimulation of protein kinase C may not only activate but also inhibit neutrophil responses is also evident from our data showing complete suppression of stimulant-induced chemokinesis (Figure 3). The protocol of the experiments dictates that the phorbol esters or OAG must be added at the same time as the chemokinetic stimulant and left in contact for the 4 h incubation period (see Methods), so we do not know whether preincubation would alter the nature of the results. However, it is likely that it represents the steady state response to prolonged protein kinase C stimulation mediated by persistently phosphorylated protein products.

Several explanations for these inhibitory effects are worth considering. One is that the inhibition of cell movement is caused by (a) enhanced cellular adhesiveness or (b) increased cell-cell adherence or (c) aggregation of the neutrophils, the last of which is a well recognized response to low doses of PMA (<10 nM) or diacylglycerols (Gallin *et al.*, 1978; Sha'afi *et al.*, 1983; O'Flaherty *et al.*, 1985). However, we did not observe cell aggregates microscopically, and peripheral neutrophils aggregate less readily than elicited neutrophils, even when stirred (Bray *et al.*, 1980).

Despite this, serious consideration should be given to the possibility that inhibition of chemokinesis occurred as a result of increased adhesiveness of the cells to the substrates through which they move. PMA has been shown by Harlin *et al.* (1985) and Anderson *et al.* (1986) to increase the expression on the neutrophil's surface of the plasma membrane associated glycoprotein complex (the 'Mac-1 glycoprotein family'). These proteins play an important role in adherence-dependent leukocyte functions such as chemotaxis and phagocytosis (see Springer & Anderson, 1986, for review). Evidence was obtained that these glycoproteins are mobilized from an intracellular pool present on the membranes of the specific granules, an interpretation compatible with the fact that PMA causes specific granule secretion. Thus treatment of the neutrophils with PMA (or other stimulants of protein kinase C) might cause a progressive increase in cellular stickiness as the specific granule membranes become incorporated into the cell membrane surface. This in turn could account for reduced cellular motility. However, it should be recognized that up-regulation of other types of surface proteins (e.g. of the high affinity FMLP receptor) may also occur as a result of secretion of the specific granules (Fletcher *et al.*, 1982), and that this is thought to accompany chemotactic movement of neutrophils and to be instrumental for it (reviewed by Gallin, 1984). Thus the end result of these mutually antagonistic processes (increased directional movement due to receptor-ligand stimulation *versus* increased adhesiveness) may depend upon the precise timing of the changes, as well as on the concentrations of the chemical stimulants.

An alternative explanation for our results is that PMA, PDBu and OAG directly reduce cell locomotion, perhaps as a result of decreases in the availability of cytoplasmic free calcium. Calcium is necessary for activation of the protein 'motor' of leukocytes via activation of calcium-dependent gelsolin and the calcium/calmodulin-dependent phosphorylation of myosin (Stossel *et al.*, 1984). Several authors have shown that stimulation of neutrophils by phorbol esters or OAG causes inhibition of agonist-induced and A23187-induced increases in cytosolic Ca^{2+} and speeds the rate of its return towards or below the pre-stimulation values (e.g. Sha'afi *et al.*, 1983; Fujita *et al.*, 1984; Schell-Frederick, 1984; Rickard & Sheterline, 1985; Naccache *et al.*, 1985; Della Bianca *et al.*, 1986), and we found this too (see Results).

At least two mechanisms for this effect on calcium are possible: activation of a calcium extruding plasma membrane pump (Lagast *et al.*, 1984; Rickard & Sheterline, 1985), or inhibition of phospholipase C-dependent phosphoinositide turnover in the PMN (Della Bianca *et al.*, 1986), leading to decreased mobilization of cytoplasmic free calcium by inositol trisphosphate. This latter substance is thought to be responsible, at least in part, for calcium mobilization from the endoplasmic reticulum in many cells (Berridge & Irvine, 1984) including neutrophils (Burgess *et al.*, 1984; Prentki *et al.*, 1984). If diacylglycerol formed *in situ* does indeed inhibit phospholipase C, then this seems a logical feedback signal to ensure transience of the receptor-mediated phosphoinositide turnover event and of the ensuing activation of protein kinase C and calcium mobilization.

As another alternative, the observed inhibition of both basal and stimulated locomotion may depend on the phosphorylation of a protein involved in cell motility. One candidate is the 20 kDa calmodulin-dependent myosin light chain kinase, whose activity is decreased by protein kinase C *in vitro* (Nishikawa *et al.*, 1983), leading to reduced activity of the actin-activated Mg-ATPase and microfilament formation. In fact, in intact neutrophils PMA causes phosphorylation of a cytoplasmic protein of approx. 20 kDa which might correspond to myosin light chain kinase (Andrews & Babior, 1983; White *et al.*, 1984), but the concentrations of PMA needed for this (16–160 nM) are greater than those causing complete inhibition of locomotion (Figure 3).

Thus the inhibitory effects of phorbol esters and OAG on chemokinesis require further clarification,

perhaps by observing cell motility directly so as to distinguish actions on cell surface adhesion from those on movement. Either way, the effects may be of relevance to the physiology of neutrophil function. We have previously pointed out (Hoult & Nourshargh, 1985) that these divergent effects on degranulation and chemokinesis following activation of the diacylglycerol/protein kinase C system can be accommodated in a simple model of neutrophil activation. It is well known that low doses of neutrophil stimulants cause chemokinesis and chemotaxis, responses which however are progressively inhibited at higher stimulant concentrations (e.g. Schiffmann *et al.*, 1975; Gallin *et al.*, 1978; Smith & Walker, 1980; Palmer *et al.*, 1980; Hoult & Nourshargh, 1985). These low doses of stimulants mobilize intracellular calcium (Korchak *et al.*, 1984; Volpi *et al.*, 1984; Rossi *et al.*, 1985; Nourshargh, 1986) and are associated with chemotactic receptor up-regulation (Gallin, 1984), whereas higher doses are needed to elicit the respiratory burst and degranulation of both specific and azurophil granules (Lehmeyer *et al.*, 1979; Korchak *et al.*, 1984; Hoult & Nourshargh, 1985; Rossi *et al.*, 1985) and phosphoinositide turnover with diacylglycerol formation (Dougherty *et al.*, 1984; Rossi *et al.*, 1985; Bradford & Rubin, 1985; but compare Volpi *et al.*, 1985). Apart from showing that mobilization of cytoplasmic calcium can occur without phosphoinositide turnover (at least in neutrophils), these results together with the present data suggest the possibility that inhibition of leukocyte movement at the higher stimulant doses (i.e. when a leukocyte has reached an inflammatory focus or source of chemotactic gradient) may result from endogenous diacylglycerol formation. This conclusion is valid regardless of whether the inhibition of movement by activation of the C-kinase is due to increased cellular adhesion or to reduced motility. The products of the activated kinase then contribute to the pro-inflammatory responses (respiratory burst, enzyme secretion, phagocytosis) of the now immobilized cell.

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