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Phorbol myristate acetate enhances human polymorphonuclear neutrophil release of granular enzymes but inhibits chemokinesis

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1 The effects of the co-carcinogenic phorbol ester, phorbol myristate acetate (PMA), on N-formyl-Met-Leu-Phe (FMLP)-induced human polymorphonuclear leukocyte chemokinesis and release of granular lysozyme and β-glucuronidase were compared with those of the inactive phorbol didecanoate (PDD).

2 Release of the enzymes was enhanced by PMA but was unaffected by PDD which also had no effect on chemokinesis.

3 In contrast, FMLP-induced chemokinesis was completely suppressed by PMA in a dose-dependent fashion (ID_{50} = 3.5 nM).

4 PMA also inhibited the FMLP-induced increase in cytoplasmic calcium level, measured by the fluorescent indicator quin-2.

5 These and other results suggest that although the diacylglycerol/protein kinase C system is involved in the positive regulation of certain neutrophil functions (degranulation and superoxide generation), if it is very powerfully stimulated, as with PMA, it has inhibitory actions on other neutrophil properties such as motility.

Introduction

Polymorphonuclear leukocytes (PMNs) exhibit a variety of cellular responses when stimulated by soluble factors such as chemotactic agents and the calcium ionophore A23187. These responses include aggregation, generation of superoxide anion radicals, release of granular proteins and enzymes and, at lower doses of stimulant, chemokinesis and chemotaxis. It is thought that phospholipase C-dependent phosphatidylinositol bisphosphate breakdown, activation of protein kinase C by diacylglycerol and a rise in the level of intracellular calcium all contribute to these effects after surface stimulation of the cells, although their relative contributions to the overall response may vary according to the nature of the stimulant.

The co-carcinogen phorbol myristate acetate (PMA) which directly activates protein kinase C (Castagna *et al.*, 1982) causes neutrophil aggregation, superoxide generation, enzyme secretion and phosphorylation of several polypeptides (see White *et al.*, 1984) but does not increase levels of intracellular calcium (Sha'afi *et al.*, 1983). However, it is not known what effects PMA may have on stimulated neutrophil locomotion (chemokinesis). This paper documents the full dose-response curves for N-formyl-Met-Leu-Phe (FMLP)-induced chemokinesis and release of granular lysozyme and β-glucuronidase enzymes from human PMNs and shows that PMA has a divergent effect on these responses, enhancing degranulation but inhibiting chemokinesis.

Methods

Suspensions of PMN from healthy non-medicated volunteers were prepared according to Dougherty *et al.* (1984) and suspended at > 95% purity and viability in Eagle's minimum essential medium buffered at pH 7.4 with 20 mM HEPES for chemokinesis assays, or in pH 7.4 phosphate buffered saline (NaCl 138 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, KCl 2.7 mM, glucose 0.1% w/v and containing 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$) for enzyme secretion experiments. Chemokinesis was measured by the agarose microdrop technique (Smith & Walker, 1980) with approximately 5×10^5 cells per well and 100 μl volumes of test solution incubated for 4 h at 37°C. Secretion of lysozyme and β-glucuronidase was determined spectrophotometrically in supernatants

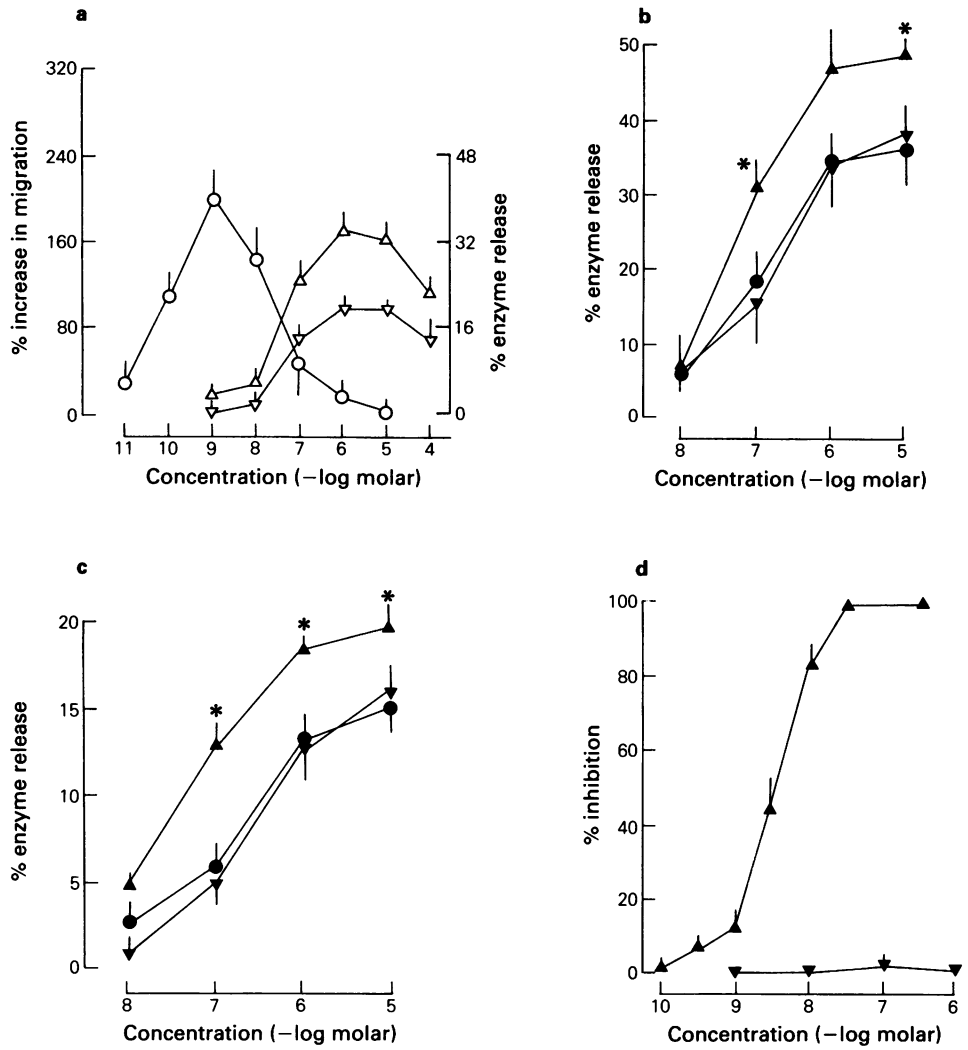

Figure 1 Human polymorphonuclear leukocyte (PMN) responses and the effects of phorbol myristate acetate (PMA) and phorbol didecanoate (PDD). (a) Chemokinesis (○), secretion of lysozyme (△) and β-glucuronidase (▽) as a function of N-formyl-Met-Leu-Phe (FMLP) concentration (results are mean values of 5, 4 and 4 separate experiments, respectively). (b and c) Effects of 10^{-8}M PMA (▲) or PDD (▼) on secretion of lysozyme (b) and β-glucuronidase (c) compared to control responses (●) to various doses of FMLP (results are mean values from 3 separate experiments and the PMA/PDD were pre-incubated for 5 min). (d) Effects of varying doses of PMA (▲) or PDD (▼) on chemokinesis induced by 10^{-9}M FMLP (3 separate experiments, phorbol esters added simultaneously). In (b) and (c) the effects of PMA in causing release of 4.3% and 2.8% of cellular lysozyme and β-glucuronidase, respectively, have been subtracted. Note that within experiments, enzyme release was assayed in duplicate tubes and chemokinesis in quadruplicate wells. Bars show s.e.mean and * indicates a value significantly different from control by Student's unpaired t test, $P < 0.05$.

obtained from cytochalasin B (5μg ml^{-1})-treated cells, incubated with varying concentrations of FMLP for 5 min at 37°C and centrifuged; the substrates used were suspensions of *Micrococcus lysodeikticus* and 4-methylumbelliferyl-β-D-glucuronide, respectively (Yuli *et al.*, 1982; Barrett & Heath, 1979). Enzyme release is expressed as percentage of total content determined in detergent-lysed cells, after correcting for spontaneous release in vehicle-treated blanks. Changes in intracellular calcium levels were measured in

cells pretreated with quin-2-tetraacetoxymethyl ester (Lancaster Synthesis, U.K.) as described by White *et al.* (1983). Phorbol esters, enzyme substrates and FMLP were purchased from Sigma Chemical Company, U.K.

Results

FMLP dose-dependently increased human PMN chemokinesis in the range 10^{-11} to 10^{-9}M, but higher concentrations progressively inhibited movement (Figure 1a). Release of granular enzymes was stimulated in the range 10^{-8} to 10^{-4}M, peaking at 10^{-6}M, a concentration that almost totally inhibited stimulated movement of the cells (Figure 1a). A higher proportion of total cellular lysozyme was released than of β-glucuronidase, reflecting their origin from (predominantly) specific and azurophil granules, respectively.

Figure 1b and c show that over the higher range of FMLP concentrations needed to elicit release of granular enzymes (10^{-8} to 10^{-5}M), PMA at 10^{-8}M enhanced enzyme release. This was significant at FMLP concentrations of 10^{-7}M and above. The analogue PDD (4α-phorbol-12,13-didecanoate, which does not activate protein kinase C, Castagna *et al.*, 1982) was not active. However, in complete contrast, PMA dose-dependently inhibited chemokinesis induced by 10^{-9}M FMLP, with an IC_{50} of 3.5 nM (Figure 1d); again, PDD was inactive.

In separate experiments, 10^{-8}M PMA (a concentration that enhances enzyme release but very strongly inhibits chemokinesis) considerably reduced the FMLP-induced increase in free intracellular calcium as detected by the quin-2 method, and also caused a reduction in the baseline level of free intracellular calcium (Figure 2).

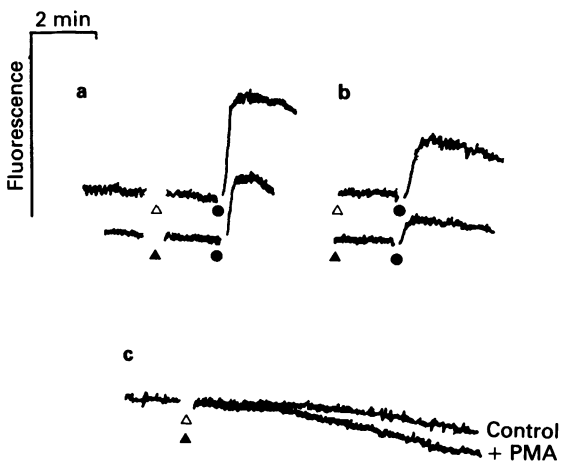

Figure 2 Effect of phorbol myristate acetate (PMA) on N-formyl-Met-Leu-Phe (FMLP)-induced increase in free intracellular calcium as detected by the quin-2 technique. The vertical line indicates fluorescence intensity in arbitrary units. In (a) and (b) effects of 10^{-8}M PMA added at ▲ (or PMA vehicle at △) are shown on the changes subsequently induced by 10^{-7}M (a) or 10^{-8}M (b) FMLP added at ●. In (c) resting cells preloaded with quin-2 were exposed at △ to PMA vehicle or at ▲ to 10^{-8}M PMA and the fluorescence was continuously monitored (excitation 339 nm, emission 492 nm) in a Perkin-Elmer model MPF-4 recording spectrophotofluorimeter at 37°C with continuous stirring. Similar results to those shown in (a) to (c) were obtained on at least 4 other occasions. In 23 different preparations of cells, the concentration of basal free intracellular calcium was 138 ± 17 nM (s.e.mean) (cf. White *et al.*, 1983; Di Virgilio *et al.*, 1984).

Discussion

Our finding that PMA inhibits FMLP-induced human PMN chemokinesis suggests that neutrophil motility is modulated in a negative way by the diacylglycerol/protein kinase C system. Additional evidence for this is: (1) that PDD did not alter chemokinesis (Figure 1d); (2) that another active co-carcinogenic phorbol ester (phorbol-12,13-dibutyrate) had similar effects to PMA; (3) that 1-oleoyl-2-acetyl glycerol also inhibited chemokinesis; (4) that a very similar profile of effects was observed if leukotriene B_4 was used to stimulate the neutrophils (points 2 to 4:S. Nourshargh and J.R.S. Hoult, unpublished experiments), and (5) that Gallin *et al.* (1978) showed that PMA at concentrations greater than 1.6 nM inhibited the ability of human neutrophils (previously allowed to degranulate and washed) to move chemotactically in a gradient of partially purified C5a.

This inhibitory effect of PMA on chemokinesis is in sharp contrast to its previously recognised ability to enhance the effects of neutrophil stimulants on other neutrophil functions such as superoxide generation and enzyme secretion occurring at higher stimulant doses (e.g., Gallin *et al.*, 1978; Kajikawa *et al.*, 1983; O'Flaherty *et al.*, 1984; Dale & Penfield, 1984; Blackwell *et al.*, 1985) and shown in Figure 1b and c. It should be noted that PMA is a very powerful stimulant of protein kinase C (e.g. Castagna *et al.*, 1982) and thus it may not mimic accurately the physiological context in which the kinase is stimulated, since this is brought about by the transient generation of diacylglycerol subsequent to the breakdown of inositol phospholipids (Nishizuka, 1984). Furthermore, there is a recent report which contradicts the general conclusion from the studies cited above, in that PMA at concentrations of 16 nM or more preincubated with rabbit neutrophils for 1 to 5 min inhibited their subsequent ability to release lysozomal and azurophil

enzymes in response to FMLP, C5a and leukotriene B_4 (Naccache *et al.*, 1985). The reasons for the discrepancy between these results with rabbit neutrophils and the other (human) studies have not yet been established, although Naccache *et al.* did find that A23187-induced degranulation was enhanced by the addition of PMA.

Nevertheless, like Naccache *et al.* (1985), we also found that PMA reduces the magnitude of the quin-2-detectable increase in intracellular calcium that occurs after triggering the PMNs with a chemokinetic dose of FMLP. This may be because PMA, and by extension protein kinase C, is responsible for activating a calcium-extruding pump mechanism, as shown in guinea-pig neutrophil plasma membranes by Lagast *et al.* (1984).

These events can be accommodated in a simple model in which activation of neutrophil movement is associated with mobilisation of intracellular calcium, needed for triggering cell motility by activating calcium-dependent gelsolin and the calcium/calmodulin-dependent phosphorylation of myosin (Southwick & Stossel, 1983), and inhibition of movement is regulated by the diacylglycerol/protein kinase C system. It is relevant that low doses of FMLP are known to mobilise intracellular calcium (see for example White *et al.*, 1983), whereas the doses needed to cause phospholipase C-dependent generation of diacylglycerol are higher and correlate with those producing degranulation (Dougherty *et al.*, 1984). Coupling of FMLP to different subcellular transduction mechanisms may also be dependent upon the fact that there are at least two affinity states of the FMLP receptor which may be interconvertible (Yuli *et al.*, 1982). Finally, it has been shown that PMA can trigger superoxide generation, exocytosis and protein phosphorylation at very low levels of intracellular calcium, suggesting that the cation is not indispensable for these neutrophil functions, provided that it is not needed for steps proximal to protein kinase C activation (Di Virgilio *et al.*, 1984).

In summary, FMLP transduction mechanisms differ in relation to chemokinesis and granular exocytosis, such that activation of protein kinase C (in these experiments achieved by adding PMA but not PDD) will inhibit movement. It has not escaped our notice that this would provide a logical mechanism for regulating human PMN function in response to a chemotactic gradient.

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