# Potentiation and inhibition of secretion from neutrophils by phorbol ester

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We demonstrate that PMA exhibits multiple effects on fMetLeuPhe and ionophore-mediated secretion from rabbit and human neutrophils: (i) Inhibition of receptor-mediated secretion appears to take place at an early stage in the stimulus-secretion coupling sequence since PMA inhibits the formation of phosphatidate. (ii) Protein phosphorylation is observed with both PMA and fMetLeuPhe and the extent of phosphorylation is additive in the presence of both stimuli. (iii) Another possible site of regulation by PMA lies distal to the rise in cytosolic $Ca^{2+}$. This is indicated by the enhancement or inhibition by PMA of the A23187-induced secretory response at low and high external $Ca^{2+}$ concentrations, respectively.

*Phorbol ester* *$Ca^{2+}$* *fMetLeuPhe* *Neutrophil* *Exocytosis* *Protein phosphorylation* *Phosphatidate*

## 1. INTRODUCTION

Activation of neutrophils with fMetLeuPhe stimulates a variety of cell functions, e.g. chemotaxis, aggregation, superoxide generation, formation of arachidonic acid derived metabolites and the exocytotic release of secretory granules [1,2]. Receptor occupation is coupled to the hydrolysis of phosphatidylinositol bisphosphate generating two intracellular messengers [3], $IP_3$ (a mediator of $Ca^{2+}$ mobilisation from intracellular stores [4]) and DG (an activator of protein kinase C [5]). It has been proposed that these two signalling pathways, both independently (in some instances) and synergistically, may be responsible for activating the ligand-induced responses of many cell types [6].

The two signalling pathways can be activated independently by the use of $Ca^{2+}$ ionophores and phorbol esters. $Ca^{2+}$ ionophores (A23187 or ionomycin) alone can activate many of the neutrophil processes including secretion from azurophilic granules [7,8]. Phorbol esters, such as PMA, which do not raise the intracellular $Ca^{2+}$ levels [9], are also effective agonists for some of the neutrophil functions due to their ability to mimic DG and so directly activate protein kinase C [5]. PMA is a good stimulus for both superoxide generation and secretion from the specific granules [10–12]. However secretion from the azurophil granules is slow and only becomes significant over a period of 30 min [10,12].

In cells responding normally to stimulation of cell surface receptors, the rise in cytosolic $Ca^{2+}$ and the generation of DG occur simultaneously. Therefore, one might expect to be able to achieve a full activation response by artificial provision of both signals at concentrations which when applied alone are insufficient. In this way it has been shown that subthreshold concentrations of $Ca^{2+}$ ionophores and PMA can synergise to cause superoxide generation [13,14], chemotaxis [15] and release from both specific [11,14] and azurophil granules [14,16–18].

*Abbreviations:* PDA, 4$\alpha$-phorbol 12,13-didecanoate; PMA, 4$\beta$-phorbol 12$\beta$-myristate 13$\alpha$-acetate; $IP_3$, inositol 1,4,5-trisphosphate; DG, diacylglycerol; fMet, formylmethionyl; PBS, NaCl (0.154 M) containing 10 mM Na phosphate (pH 7)

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Here we present evidence that PMA has multiple effects on secretion of azurophil granules stimulated by either the agonist fMetLeuPhe or the $Ca^{2+}$ ionophore. The combination of PMA and $Ca^{2+}$ ionophore can lead to potentiation or inhibition of secretion. PMA also inhibits the formation of phosphatidate due to fMetLeuPhe. We conclude that PMA not only inhibits events subsequent to increases in cytosol $Ca^{2+}$ but also inhibits at the level of the receptor.

## 2. MATERIALS AND METHODS

Rabbit peritoneal exudate and human peripheral neutrophils were prepared as described [2,19]. They were suspended at $10^7$ cells·ml$^{-1}$ in a buffered salt solution (pH 7.2) which comprised 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (unless otherwise stated), 20 mM Hepes, 5.6 mM glucose and 1 mg·ml$^{-1}$ bovine serum albumin. The cells were incubated for 15 min at 37°C before addition of cytochalasin B (final concentration 5 $\mu$g·ml$^{-1}$). After 2 min the cells were transferred to solutions containing appropriate concentrations of ionophore A23187, fMetLeuPhe or PMA, either individually or in combination. In some experiments the cells were pretreated with PMA (for the times indicated in the legends to figs 1 and 2) before transfer to A23187 or fMetLeuPhe. After the times indicated, the reactions were quenched by addition of ice-cold PBS. Following centrifugation, samples of the supernatants were removed for measurement of secreted $\beta$-glucuronidase [2]. The experiments presented in figs 1–3 and tables 1 and 3 were done in duplicate and the determinations did not differ by more than 5%.

For the experiments of fig.3 and table 2, the rabbit neutrophils were resuspended at $10^7$ cells·ml$^{-1}$ in buffer made up as described but containing bovine serum albumin at 0.25 mg·ml$^{-1}$. They were incubated for 1 h at 37°C with 1 mCi·ml$^{-1}$ [$^{32}$P]phosphate. The cells were then washed twice, cytochalasin B was added and after 2 min the cells were transferred to an equal volume of buffer containing stimulating ligands as indicated. For the analysis of protein phosphorylation, duplicate samples were quenched after 1 min by addition of 5% (final concentration) trichloroacetic acid. Triplicate samples were quenched with ice-cold PBS for the determination of enzyme secretion and phospholipid analysis. The lipids were extracted from the pelleted cells with chloroform/methanol [19] and the samples analysed by TLC as described [20]. SDS-polyacrylamide gel electrophoresis was carried out in a discontinuous system using a 10% separating gel (pH 8.8) and 3% stacking gel (pH 6.8) as described by Laemmli [21]. Autoradiographs of the stained and dried gels were analysed on a Joyce-Loebel Chromoscan.

Molecular mass markers (12–78000 Da range) were obtained from BDH, Poole, England. All other reagents were of the best analytical grades available and were obtained from BCL (Lewes) or Sigma (Poole).

## 3. RESULTS

We preincubated rabbit neutrophils with $10^{-7}$ M PMA for 5 min and then challenged the cells with A23187 in the presence of 50 $\mu$M or 1 mM $Ca^{2+}$. The rationale for comparing different external $Ca^{2+}$ was to induce submaximal and maximal degrees of secretion [7]. A typical experiment is presented in fig.1, and results from four individual experiments (± SE) are pooled in table 1. In the presence of 50 $\mu$M $Ca^{2+}$, the maximal extent of secretion due to A23187 alone (in the range

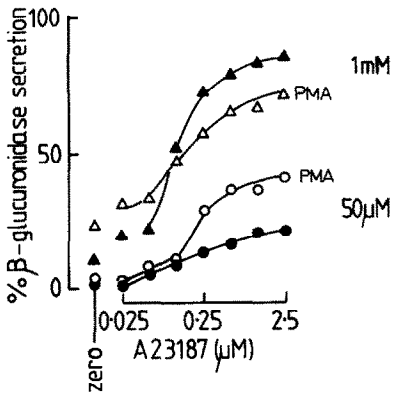


Fig.1. Relationship of extracellular calcium to the effects of PMA on A23187-mediated $\beta$-glucuronidase secretion. Rabbit neutrophils were preincubated in the presence (open symbols) and absence (solid symbols) of $10^{-7}$ M PMA for 3 min before transfer to solutions containing A23187 at concentrations indicated and calcium at 50 $\mu$M (●,○) and 1 mM (▲, △) for a further 3 min at 37°C.

Table 1

Enhancement and inhibition of A23187-induced β-glucuronidase secretion by PMA

| | % β-glucuronidase secretion | |
|---|---|---|
| Extracellular $Ca^{2+}$: | 50 μM | 1 mM |
| A23187 | 36 ± 6 (4) | 63 ± 6 (4) |
| Plus PMA $10^{-7}$ M | 44 ± 4 (4) | 38 ± 4 (4) |

The ionophore was used at 2.5 μM, a concentration sufficient to induce maximal secretion at any concentration of $Ca^{2+}$. Results expressed as means ± SE (*n*, number of separate experiments)

0.025–2.5 μM) is moderate and can be potentiated by PMA. In contrast, the higher level of secretion due to 1 mM $Ca^{2+}$ is inhibited by PMA.

We next examined the effect of PMA on fMetLeuPhe stimulated secretion. Whilst the combination of PMA with subthreshold levels of fMetLeuPhe causes a slight enhancement of β-glucuronidase secretion, PMA inhibits the secretion due to saturating concentrations of the ligand (fig.2a). This inhibition of receptor-mediated degranulation is even more extensive if the cells are preincubated with PMA before challenging with fMetLeuPhe (fig.2b). The inactive phorbol ester PDA not only fails to enhance any β-glucuronidase secretion due to low concentrations of fMetLeuPhe but also has no inhibitory effect at higher ligand concentrations. Fig.2b also shows that the inhibition by PMA of fMetLeuPhe-mediated activation is common to both rabbit peritoneal and human peripheral neutrophils.

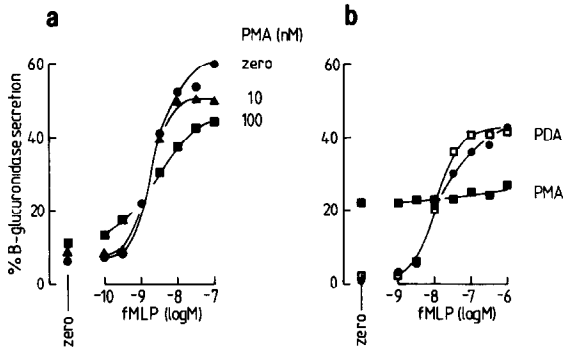


Fig.2. Inhibition by PMA of β-glucuronidase secretion due to fMetLeuPhe in rabbit and human neutrophils. (a) Rabbit neutrophils were stimulated with different concentrations of fMetLeuPhe for 5 min in the absence (●) and presence of $10^{-8}$ M (▲) and $10^{-7}$ M PMA (■). (b) Human neutrophils were preincubated with either buffer (●), PMA (■) or PDA (□) (both at $10^{-7}$ M) for 15 min at 37°C before being stimulated with different concentrations of fMetLeuPhe for 5 min.

To determine at what level PMA inhibits fMetLeuPhe-induced responses we investigated its effect on phosphatidate formation. This allowed us to monitor the effect of PMA on the generation of DG from the hydrolysed inositol lipids, a process closely associated with the initial events in the generation of second messenger molecules. Rabbit neutrophils prelabelled to equilibrium with $^{32}P$ were treated with PMA and fMetLeuPhe, either individually or in combination. The β-glucuronidase release stimulated by the combination of fMetLeuPhe ($10^{-7}$ M) and PMA ($10^{-7}$ M) was inhibited by approx. 40% compared to the release stimulated by the ligand alone. This correlates with the inhibition by PMA of fMetLeuPhe-induced formation of phosphatidate (table 2).

As PMA is an activator of protein kinase C we considered the possibility that the inhibition due to PMA was caused by phosphorylation of some essential regulatory protein. This was examined by determining the profile of $^{32}P$ incorporation into proteins from cells treated for 1 min with either PMA, fMetLeuPhe or their combination. Both PMA and fMetLeuPhe induced similar profiles of protein phosphorylation, the difference being in

Table 2

Inhibition by PMA of β-glucuronidase secretion and phosphatidate formation due to fMetLeuPhe

| | β-Glucuronidase (% secretion) | Phosphatidate formation (% of control) |
|---|---|---|
| Control | 0 | (100) |
| fMetLeuPhe ($10^{-7}$ M) | 68 ± 10 (6) | 192 ± 16 (7) |
| PMA ($10^{-7}$ M) | 5 ± 1 (6) | 93 ± 6 (7) |
| fMetLeuPhe plus PMA | 48 ± 8 (6) | 147 ± 16 (7) |

Results expressed as means ± SE (*n*, number of separate experiments)

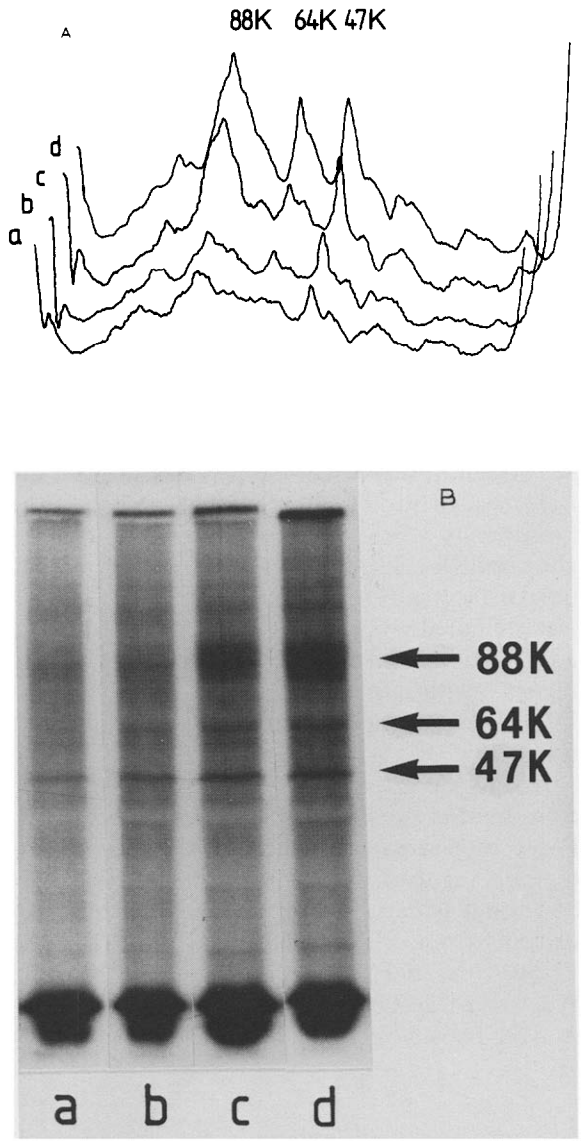


Fig.3. Protein phosphorylation in rabbit neutrophils after stimulation with PMA and fMetLeuPhe, individually and in combination. Rabbit neutrophils, labelled with [$^{32}$P]phosphate, were stimulated for 1 min at 37°C. (a) Control; (b) $10^{-7}$ M PMA; (c) $10^{-7}$ M fMetLeuPhe; (d) $10^{-7}$ M PMA plus $10^{-7}$ M fMetLeuPhe. After quenching with ice-cold 5% trichloroacetic acid the precipitates were pelleted and then washed with PBS before solubilisation in SDS sample buffer. After SDS-polyacrylamide gel electrophoresis the dried gel was autoradiographed for 3 days (A) and the lanes scanned with a Joyce-Loebl Chromoscan (B). The molecular masses of the bands of interest are indicated.

Table 3

Increase in incorporation of [$^{32}$P]phosphate into neutrophil proteins on stimulation with fMetLeuPhe and PMA individually and in combination

| | 88 kDa | 64 kDa | 47 kDa |
|---|---|---|---|
| Control | 100 | 100 | 100 |
| fMetLeuPhe | 163 ± 4 | 140 ± 10 | 138 ± 12 |
| PMA | 121 ± 4 | 122 ± 6 | 140 ± 3 |
| fMetLeuPhe plus PMA | 207 ± 6 | 158 ± 13 | 201 ± 24 |

Data represent percentage increase of peak areas (means ± SE; $n$ = 6 separate experiments)

the degree of phosphorylation found in individual bands (fig.3, table 3). The combination of the stimuli led to a particularly marked enhancement in the phosphorylation of proteins of $M_r$ 88000, 64000 and 47000.

## 4. DISCUSSION

The pattern of fMetLeuPhe-mediated secretion of azurophil granules can be mimicked under certain circumstances by artificial enhancement of cytosol $Ca^{2+}$ and activation of protein kinase C. The potentiation which we have observed is rather modest and is comparable to that reported by some [16,17] but less than that recorded by others [14,18]. Activation of protein kinase C by PMA can also lead to inhibition of the fMetLeuPhe-mediated and the $Ca^{2+}$-mediated responses. It would appear that the inhibition of receptor-mediated events by PMA takes place at a stage in the secretory process prior to the elevation of cytosol $Ca^{2+}$. This conclusion is supported by the observation that phosphatidate production, and hence presumably phosphatidylinositol bisphosphate hydrolysis, is also inhibited by PMA. It has been reported that PMA can inhibit the receptor-mediated rise in cytosolic $Ca^{2+}$ [18,22] corroborating the above conclusion that it interferes with the generation of $IP_3$. Inhibition of receptor-stimulated inositol lipid metabolism by PMA has been reported for PC12 cells [23], platelets [24,25], astrocytoma cells [26] and smooth muscle [27–29] and may reflect a common feedback control mechanism in $Ca^{2+}$-mobilising receptors.

To inhibit the hydrolysis of phosphatidylinositol

bisphosphate it may be necessary to uncouple the receptor from its catalytic unit, polyphosphoinositide phosphodiesterase, and this could be achieved by PMA-induced phosphorylation of either the receptor itself or the guanine nucleotide regulatory protein ($N_P$) which couples the two [30]. Enhanced phosphorylation of several protein bands occurs when rabbit neutrophils are challenged with the combination of fMetLeuPhe and PMA (fig.3) and one of these has an $M_r$ of 64000 which is similar to the reported $M_r$ of the formylpeptide receptor [31–33]. Furthermore, PMA-induced phosphorylation of receptors [34–36] and G-proteins [37–39] as a means of modulating signal transduction has now been reported for a number of different systems and may reflect a role for protein kinase C in the activation and termination of cellular secretory processes.

PMA may also act at a second site in the train of events leading to exocytosis of azurophil granules since it is an inhibitor of ionophore-induced secretion and the secretion due to $Ca^{2+}$ buffers introduced into permeabilised neutrophils [40]. Alternatively it is possible that PMA prevents the formation of the products of arachidonic acid metabolism arising from ionophore stimulation. A23187 can stimulate production of leukotriene $B_4$ [41], PAF [42] (both stimulatory for neutrophils) and 5-HETE which potentiates their actions [43]. In this case the inhibition by PMA of the azurophil granule secretion induced by optimal levels of A23187 and $Ca^{2+}$ may parallel the inhibition by PMA of fMetLeuPhe-induced secretion. Against this, there is evidence that the secretion due to A23187 is quite independent of leukotriene [44] or 5-HETE [45] synthesis.

In conclusion, we find that PMA can either synergise with or inhibit secretion of $\beta$-glucuronidase, depending on the conditions. In particular, it enhances secretion when the extent of $Ca^{2+}$ elevation due to the ionophore is limited, but becomes inhibitory in the face of higher levels of cytosol $Ca^{2+}$. We also find that PMA has effects prior to the rise in cytosol $Ca^{2+}$ since the inhibition of fMetLeuPhe-mediated $\beta$-glucuronidase secretion correlates with an equivalent inhibition of phosphatidate formation and hence, presumably $IP_3$ generation. Whether all the effects of PMA can be attributed to its ability to activate protein kinase C is not known.

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