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Does protein kinase C control receptor-mediated phagocytosis in human neutrophils?

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It was previously demonstrated that C3bi- but not IgG-mediated phagocytosis in human neutrophils is a Ca^{2+}-independent process [(1985) Nature 315, 509–511]. The objective of this study was to elucidate the nature of an additional messenger signal(s) that may be involved in receptor-mediated phagocytosis in these cells. The C3bi- and IgG-mediated phagocytic ability of neutrophils was inhibited by forskolin, a potent activator of adenylate cyclase. It was found that forskolin induced a 3-fold increase in cAMP levels and simultaneously reduced the uptake of both C3bi- and IgG-opsonized particles by 65%. This inhibition was reversed by exposure to either phorbol myristate acetate (PMA) or diacylglycerol (OAG), which are both activators of protein kinase C, but not by the inactive analog 4α-PMA. PMA could also restore the phagocytic ability of neutrophils with an experimentally impaired calcium response. These results suggest that activation of protein kinase C might be an important transducing signal controlling receptor-mediated phagocytosis in human neutrophils.

Protein kinase C; Phagocytosis; (Human neutrophil)

1. INTRODUCTION

The phagocytic process of human neutrophils is triggered by two major receptor-mediated mechanisms: one which recognizes the C3bi-fragment of the complement system and another which recognizes the Fc domain of immunoglobulin G [1]. The nature of the mechanisms that control phagocytosis in human neutrophils is not clear. On the other hand, soluble stimuli, including chemotactic factors, induce an immediately increased activity of phospholipase C. This enzyme causes hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in a corresponding increase in the production of two well-known second messengers, namely, inositol 1,4,5-trisphosphate (Ins (1,4,5)P_3) and 1,2-diacylglycerol [2–6]. The former induces mobilization of intracellular Ca^{2+}, whereas the latter binds to and activates protein kinase C [2,3].

Protein kinase C is generally considered to act in concert with calcium as a transducing signal for a number of chemotactic stimuli.

In two independent studies on C3bi- and IgG-mediated phagocytosis a concomitant elevation in the concentration of cytosolic free calcium has been clearly demonstrated [7–9]. An increase in concentration of cytosolic free calcium appears to be required for ingestion of IgG-coated particles by human neutrophils, whereas an elevation of calcium is not at all essential for the engulfment of C3bi-opsonized particles [8]. Because the process of engulfment can be accomplished in cells with a

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Abbreviations: fMet-Leu-Phe, formylmethionylleucylphenylalanine; $InsP_3$, inositol trisphosphate; 1,4,5-$InsP_3$, inositol 1,4,5-trisphosphate; PMA, phorbol 12-myristate 13-acetate; 4α-PMA, 4α-phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetylglycerol; $[Ca^{2+}]_i$, cytosolic free calcium concentration

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reduced concentration of cytosolic free calcium (less than 20 nM), the involvement of an alternative messenger signal participating in the initiation and regulation of receptor-mediated phagocytosis is necessary. Based on these and other observations, it is at present suspected that increased turnover of the inositol cycle and activation of protein kinase C may be involved in receptor-mediated phagocytosis [10,11].

Consequently, the objective of this study was to investigate whether protein kinase C can function as a transducing signal for receptor-mediated phagocytosis in human neutrophils.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Materials and their sources were as follows, forskolin (Calbiochem, La Jolla, CA); quin2/AM, quin2 and prostaglandin E_1 (Amersham, Bucks); fMet-Leu-Phe, OAG, and PMA (Sigma, St. Louis, MO); 4α-PMA (LC Serv. Corp., Woburn, MA); dextran, and Ficoll-Hypaque (Pharmacia, Uppsala).

2.2. *Preparation of cells*

Blood was obtained from healthy volunteers and collected in heparin-containing tubes. Whole blood was allowed to sediment in dextran, after which the neutrophils were isolated according to Böyum [12]. Contaminating erythrocytes were eliminated by 20 s hypotonic lysis in distilled water, after which the cells were washed twice before being suspended in a calcium-containing medium [this medium included: 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 1 mM $NaHPO_4$, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes (pH 7.4)].

2.3. *Determination of cAMP content*

Human neutrophils (10^7/ml) were incubated with forskolin (100 μM) for various periods of time at 37°C. The reactions were terminated by adding ice-cold ethanol (final concentration 65%). The different samples were then allowed to settle for 1 h at −20°C, after which the different supernatants containing cAMP were collected. The precipitated materials were washed in fresh 65% ice-cold ethanol to ensure that the extraction of cAMP was as complete as possible. The supernatants were then centrifuged at 2000 × g for 15 min at 4°C, and the precipitates were discarded. The remaining supernatants were then evaporated under nitrogen, after which the cAMP content was determined with a commercial cAMP assay kit (Amersham).

2.4. *Determination of phagocytosis*

Phagocytosis of FITC-labelled yeast particles was monitored by a fluorescence quenching method [8,13], in which the extracellular particles are quenched by addition of trypan blue. The IgG-coated particles were opsonized by incubating yeast with purified anti-yeast IgG (24 μg/ml) in the presence of 20% heat-inactivated (56°C, 30 min) human serum; C3bi-coated particles were opsonized by incubating the yeast with 20% fresh human serum. All particles were opsonized by incubation at 37°C for 30 min and washed before use. Phagocytic activity for each condition was determined by calculating the number of yeast particles ingested per 100 cells.

3. RESULTS AND DISCUSSION

It is well known that cAMP can inhibit receptor-induced responses, including motile events such as chemotaxis and phagocytosis, in neutrophils [14–16]. Here, forskolin, a potent activator of adenylate cyclase [17], was used to raise the intracellular content of cAMP. Control cells contained 560 fmol cAMP/10^6 cells. 10 min after the addition of forskolin, the cellular content of cAMP was increased 3-fold (312%, mean of 3 experiments). This level was maintained for at least 50 min, during which various experimental measurements were made. The forskolin-induced rise in cellular cAMP resulted in a 60–65% reduction of both C3bi- and IgG-mediated phagocytosis (figs 1,2). Prostaglandin E_1 (10 μM), whose effects are mediated by cAMP [18,19], inhibited IgG- and C3bi-mediated phagocytosis 69 and 45% (n = 3), respectively. Therefore, it seems reasonable to assume that the observed forskolin-induced inhibition of phagocytosis is mediated by an elevation of cellular cAMP levels. However, it is not known whether the effects of cAMP on receptor-mediated phagocytosis are mediated by: (i) phosphorylation and inactivation of a pertinent receptor; and/or (ii) phosphorylation and inhibition of a cytoskeletal protein; and/or (iii) inhibition of phospholipase C and suppression of second messenger levels. The last alternative is supported by the fact that elevation of cAMP leads to the reduced formation of receptor-mediated intracellular messengers from the phosphoinositide cycle in thrombocytes and human neutrophils [20,21]. In addition, we have recently been able to demonstrate that forskolin inhibited C3bi- and IgG-mediated formation of second messengers derived from the inositol cycle in human neutrophils (Fällman et al., unpublished). It is therefore suggestive that the PMA- and OAG-induced restoration of receptor-mediated phagocytosis (figs 1,2) may best be explained by the postulate that PMA and OAG substitute for the endogenously derived diacylglycerol in activating protein kinase C. This role for protein kinase C is further supported by the observation that 4α-

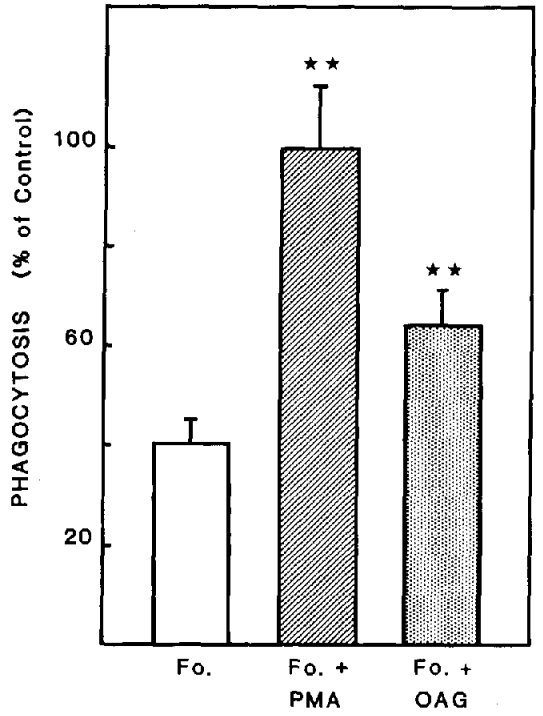

Fig.1. Restoring effects of PMA and OAG on forskolin-inhibited phagocytosis of C3bi-particles in human neutrophils. Cells were pre-incubated with forskolin (100 μM) for 30 min at 37°C. This concentration of forskolin was also maintained during the 20 min incubation with C3bi-opsonized yeast particles, which was followed by counting of the number of phagocytosed particles. In experiments studying the modulatory effects of PMA (10^{-7} M , hatched bar) and OAG (10^{-4} M, dotted bar), the additives were given during the last 10 min of the pre-incubation with forskolin and also maintained during the subsequent incubation with C3bi-opsonized yeast particles. Values given are means ± SE of 6–8 separate experiments. The 100% level for C3bi-mediated phagocytosis was 186 ± 20 yeast particles/100 cells. PMA or OAG alone did not affect C3bi-mediated phagocytosis; 97 ± 6 and 112 ± 8% of control, respectively. Statistically significant modulation by PMA and OAG is indicated. The statistical test was performed using Student's *t*-test for paired samples. ** $P < 0.01$.

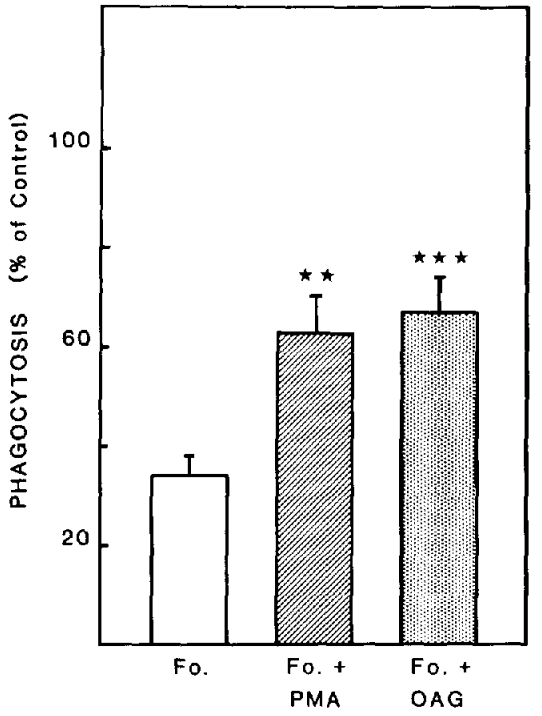

Fig.2. Restoring effects of PMA and OAG on forskolin-inhibited phagocytosis of IgG-particles in human neutrophils. Cells were pre-incubated with forskolin (100 μM) for 30 min at 37°C. This concentration of forskolin was also maintained during the 20 min incubation with IgG-opsonized yeast particles, which was followed by counting the number of phagocytosed particles. In experiments studying the modulatory effects of PMA (10^{-7} M, hatched bar) and OAG (10^{-4} M, dotted bar), the additives were given during the last 10 min of the pre-incubation and also maintained during the subsequent incubation with opsonized yeast particles. Values given are means ± SE of 6–9 separate experiments. The 100% level for IgG-mediated phagocytosis was 201 ± 30 yeast particles/100 cells. PMA alone only modestly affected IgG-mediated phagocytosis (73 ± 7% of control), whereas OAG alone had no significant effect (113 ± 9% of control). Statistically significant modulations by PMA and OAG are indicated. The statistical tests were performed using Student's *t*-test for paired samples. ** $P < 0.01$; *** $P < 0.001$.

PMA, a phorbol ester that does not activate protein kinase C, had no effect on either IgG- or C3bi-mediated phagocytosis (not shown).

The data presented in fig.3 confirm our previous observations that, in human neutrophils, IgG- but not C3bi-mediated phagocytosis is a calcium-dependent process [8]. However, even more important is the observation that phorbol ester-induced activation of protein kinase C could significantly restore the phagocytic capacity of IgG-opsonized particles in cells with an impaired $[Ca^{2+}]_i$ response (fig.3). In addition to what has been discussed above, these data indicate that protein kinase C could also take over the control of a process that is normally regulated via $[Ca^{2+}]_i$ changes. These observations are, consequently, in agreement with the general idea of the synergistic actions of a raised concentration of cytosolic free calcium on

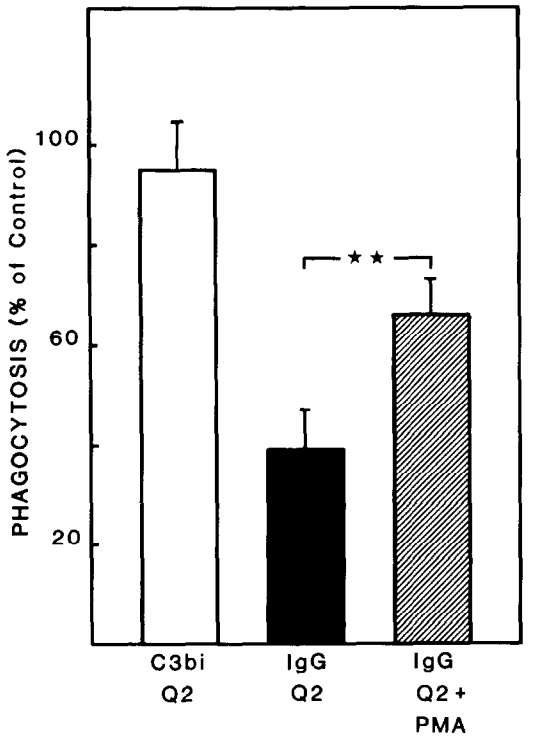

Fig.3. Effects of quin-2 buffering and PMA on C3bi- (open bar) and IgG-mediated (closed bar) phagocytosis in human neutrophils. Incubations with quin2/AM (100 μM diluted to 20 μM after 10 min) were performed for a total of 60 min at 37°C. This was followed by washing and incubation with opsonized yeast particles for 20 min at 37°C and then counting of the number of phagocytosed particles. In the situation where the modulatory effects of PMA (10^{-7} M, hatched bar) were studied, the additive was given during the last 10 min of the quin-2 loading and also maintained during the subsequent incubation with opsonized yeast particles. Values given are means ± SE of 6 separate experiments. Statistically significant modulation by PMA is indicated. The statistical tests were performed using Student's *t*-test for paired samples. ** $P < 0.01$.

the one hand and activation of protein kinase C on the other [2,4].

Although protein kinase C catalyzes the phosphorylation of several proteins rather than just one, the finding by Vaux and Gordon [22] that a 19 kDa protein in macrophages is phosphorylated, either as a result of phagocytosis or by exposure to PMA, lends further support for the view, suggested here, that protein kinase C activation is involved in the transduction mechanism that regulates receptor-mediated phagocytosis. At present we cannot say whether protein kinase C controls receptor-mediated phagocytosis directly via phosphorylation of a certain target protein(s) or indirectly via stimulation of phospholipase A_2 [23].

PMA exposure has been shown to down-regulate cell surface receptors by enhanced endocytosis [24–27]. However, down-regulation of receptors for C3bi did not have any significant effect on the phagocytic capacity of C3bi-opsonized particles in human neutrophils [24]. This finding is in agreement with the present demonstration that PMA exposure alone did not impair C3bi-mediated phagocytosis (legend to fig.1). However, the slight inhibition of PMA on IgG-mediated phagocytosis (legend to fig.2) could possibly be due to an inadequate number of cell surface receptors following PMA exposure.

The observations made in this study suggest that activation of protein kinase C can control receptor-mediated engulfment in human neutrophils. This is of particular importance, since we and others have shown that receptor-mediated phagocytosis can occur in the absence of calcium [8,9].

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