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1,2-Diacylglycerol accumulation in human neutrophils does not correlate with respiratory burst activation

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Measurements of the level of 1,2-diacylglycerol (1,2-DG) during activation of the respiratory burst of human neutrophils by formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence of platelet-activating factor (PAF) or by opsonized particles show that a correlation between accumulation of 1,2-DG and O_2 consumption does not exist. Inhibition of protein kinase C activity with staurosporine before addition of opsonized particles demonstrates that the first phase of the respiratory burst is not inhibited, whereas the second phase, which is accompanied by a rise in the content of 1,2-DG, is strongly inhibited. This study indicates that accumulation of 1,2-DG cannot be the sole signal for the initiation of the respiratory burst in human neutrophils.

Neutrophil; Respiratory burst; Diacylglycerol, 1,2-; Serum-treated zymosan

1. INTRODUCTION

Phagocytosis and killing of bacteria by neutrophils are important mechanisms in the host defense against invading micro-organisms. In vitro, opsonized particles are well phagocytized by neutrophils [1]. Upon phagocytosis, a respiratory burst is generated [2,3], which is mediated by a membrane-bound NADPH oxidase [2]. In spite of the fact that opsonized particles are strong activators of human neutrophils, little is known about the signal(s) mediating this response. Several authors have speculated on the importance of the activation of a phospholipase C acting on phosphatidylinositol bisphosphate (PIP_2) during activation of neutrophils and the subsequent production of the second messengers inositol 1,4,5-trisphosphate (Ins (1,4,5)P_3) and 1,2-diacylglycerol (1,2-DG) [4,5,6]. Intracellular 1,2-DG resulting from this hydrolysis might be, in analogy with the action of phorbol esters, responsible for the activation of the respiratory burst via activation of protein kinase C (PK-C) [7].

The aim of our study was to determine whether the accumulation of 1,2-DG in human neutrophils in response to physiological stimuli correlates with the activation of the respiratory burst in these cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Formyl-methionyl-leucyl-phenylalanine (fMLP) and cytochalasin B (Sigma, MO) and staurosporine (Boehringer, Mannheim) were dissolved in dimethyl sulfoxide (DMSO) at 1000-times the final concentration for cell incubations, and stored at −20°C. Serum-treated zymosan (STZ) was prepared as described [3]. Platelet-activating factor (β-acetyl-γ-*O*-hexadecyl-L-α-phosphatidylcholine) was obtained from Sigma and diacyl-glycerol kinase from *E. coli* was purchased from Lipidex (West Field, NJ). All other chemicals were reagent grade. 'Incubation medium' for the cell incubations contained 132 mM NaCl, 6.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) human albumin, pH 7.4.

2.2. *Cell isolation*

Blood was obtained from healthy volunteers. Granulocytes were purified from the buffy coat of 500 ml blood an-

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ticoagulated with 0.4% (w/v) trisodium citrate (pH 7.4), as in [8]. After isolation, cells were suspended in incubation medium and kept at room temperature. All experiments were performed within 3 h after isolation of the cells.

2.3. *Measurement of oxygen uptake and 1,2-diacylglycerol*

Oxygen uptake was measured at 37°C with an oxygen electrode as described before [8]. Accumulation of 1,2-DG was measured by conversion of 1,2-DG present in cell extracts to [^{32}P]phosphatidic acid with DG kinase, exactly as described [9,10].

3. RESULTS

3.1. *Measurement of 1,2-diacylglycerol levels in human neutrophils upon stimulation of the respiratory burst*

Addition of the chemotactic peptide fMLP to human neutrophils results in activation of the respiratory burst (see e.g. table 1). Despite the relatively high concentration of fMLP added (1 μM) to obtain a maximal oxidative response, the respiratory burst activity is much less than that induced by phorbol esters or opsonized particles (see below). The fMLP-induced respiratory burst can, however, be markedly enhanced by pretreatment of the neutrophils with platelet-activating factor (PAF) [11,12]. We have measured changes in the level of 1,2-DG induced by fMLP, in both control and PAF-primed neutrophils. Samples for 1,2-DG accumulation were taken 60 s after fMLP addition. Addition of fMLP (1 μM) to neutrophils resulted in a 2-fold increase in the level of 1,2-DG (fig.1). Treatment of neutrophils with PAF also resulted in a 2-fold increase in 1,2-DG (fig.1), without concomitant activation of the respiratory burst (table 1 and [11,12]). The fMLP-induced 1,2-DG accumulation was only slightly higher in PAF-treated cells than in untreated cells, in contrast to the pronounced increase in respiratory burst activity observed in these cells (table 1). For comparison, we also measured 1,2-DG levels in neutrophils pretreated with the non-physiological priming agent cytochalasin B (fig.1). Addition of fMLP to these neutrophils resulted in a pronounced accumulation of 1,2-DG, as shown previously by Rider and Niedel [10].

Table 1

Priming by PAF of the fMLP-induced respiratory burst

	− fMLP	+ fMLP (1 μM)
Primer		
Control	0.2 ± 0.1	1.8 ± 0.3
PAF (1 μM)	0.3 ± 0.1	7.2 ± 0.4

Rates of oxygen uptake are expressed as nmol O_2/10^{6}cells per min (means ± SE, n = 5)

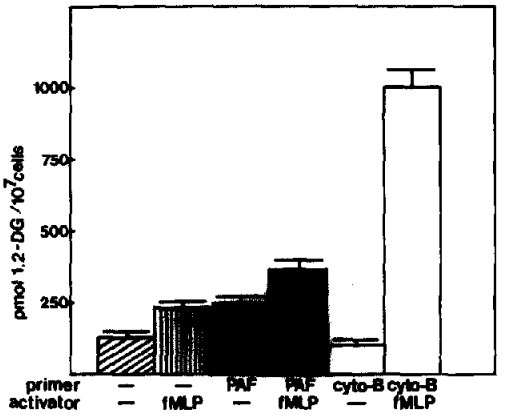

Fig.1. Accumulation of 1,2-diacylglycerol (1,2-DG) in human neutrophils induced by fMLP under resting and primed conditions. After 5 min preincubation of the cells at 37°C, platelet-activating-factor (PAF, 1 μM) or cytochalasin B (5 μg/ml) were added, and the incubation was continued for 2 or 5 min, respectively. Subsequently, fMLP (1 μM) was added and the reaction was stopped after 60 s by adding chloroform/methanol (see section 2). Results are expressed as means ± SE of three different experiments.

Addition of opsonized particles, such as STZ, at a high particle-to-cell ratio (about 70:1) results in a maximal respiratory burst activity without a requirement for priming. 1 min after addition of the opsonized particles, the respiratory burst activity had reached 70% of its maximal value as deduced by computer evaluation (fig.2). However, during the first minute after addition of STZ no net accumulation of 1,2-DG took place (fig.3). After 1 min, the amount of 1,2-DG started to increase until a 3-fold increase was obtained after 5 min.

Apparently, when fMLP or opsonized particles are used to activate the respiratory burst, a clear correlation between 1,2-DG accumulation and respiratory burst activity cannot always be demonstrated. Involvement of protein kinase C activation under these conditions seems therefore unlikely. In subsequent experiments we sought to substantiate this idea by investigating respiratory burst activation in the presence of a protein kinase C inhibitor.

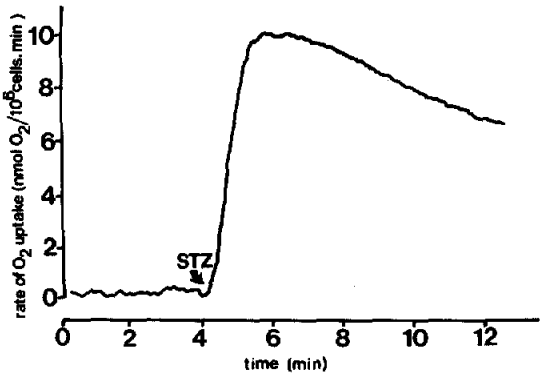

Fig.2. Computer evaluation of the time course of the rate of oxygen uptake by human neutrophils after activation with STZ (1 mg/ml). The results shown are representative for 5 different experiments.

3.2. *Influence of the PKC inhibitor staurosporine on the activation of the respiratory burst*

The microbial alkaloid staurosporine has been found to be a potent inhibitor of protein kinase C [13,14]. As shown in fig.4, the respiratory burst induced by an optimal dose of PMA (100 ng/ml) was inhibited by preincubation with staurosporine, in a dose-dependent fashion. In contrast, the fMLP response primed by PAF was only partially influenced by the presence of 200 nM staurosporine, a dose that completely blocked the PMA response.

Preincubation of human neutrophils with 200 nM staurosporine did not inhibit the first

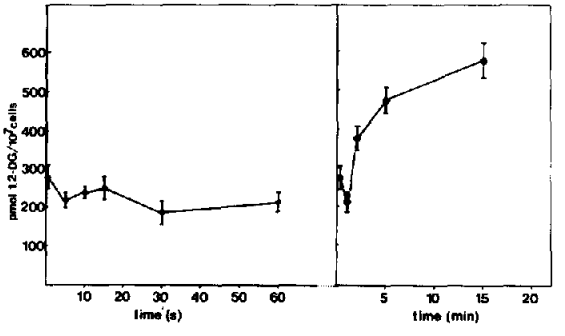

Fig.3. Time course of the STZ-induced accumulation of 1,2-DG. After 5 min preincubation of the cells at 37°C, STZ (1 mg/ml) was added to the cell suspension. Samples were taken at the times indicated and added to chloroform/methanol (see section 2). Results are expressed as means ± SE of three different experiments.

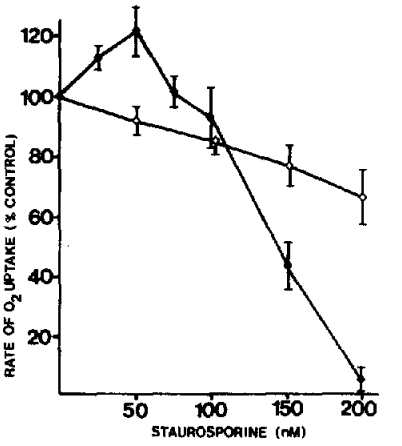

Fig.4. Effect of staurosporine on the oxygen uptake induced by the addition of PMA to resting cells or fMLP to PAF-primed cells. In the case of activation with PMA (100 ng/ml, ◆—◆), cells were preincubated for 5 min at 37°C before staurosporine was added. 2 min later, PMA was added and the maximal rate of oxygen uptake was determined. For activation with fMLP (1 μM, ◇—◇), cells were successively preincubated for 5 min at 37°C, 2 min with PAF (1 μM) and 2 min with staurosporine. Subsequently, fMLP was added and the maximal rate of oxygen uptake was determined. Results are expressed as percentage of the rates obtained in the absence of staurosporine (5.2 ± 0.5 nmol $O_2/10^6$ cells per min for PMA activation; 7.7 ± 0.5 nmol $O_2/10^6$ cells per min for fMLP activation and represent means ± SE of three different experiments).

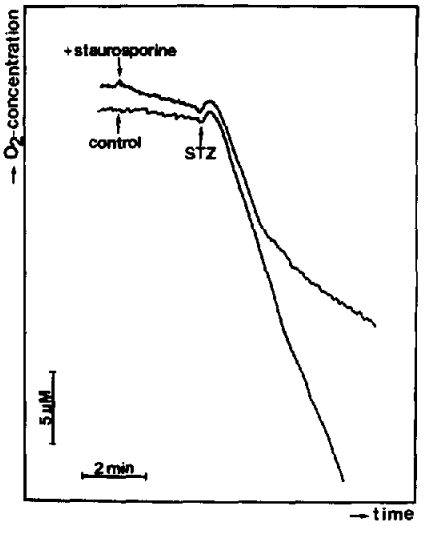

Fig.5. Effect of preincubation of neutrophils with staurosporine on the time course of the STZ-induced oxygen uptake. Cells were preincubated for 5 min at 37°C and 2 min with staurosporine (200 nM) before STZ (1 mg/ml) was added. The trace expressed is representative for 5 experiments.

phase of the respiratory burst induced by STZ (see fig.5). However, after a 1–2 min period, the STZ-induced response became markedly inhibited by staurosporine, resulting in a characteristic 'bend' in the time course.

4. DISCUSSION

1,2-DG is liberated during cell activation not only from PIP_2, but also from other sources, e.g. phosphatidylcholine [15,16]. Therefore, it is important to measure the mass of accumulated 1,2-DG to study the importance of this second messenger in the activation of the respiratory burst rather than Ins (1,4,5)P_3 or changes in Ca^{2+} homeostasis, as an indirect measure.

Our results indicate that accumulation of mass 1,2-DG from sources other than PIP_2 may occur, especially during the second phase of the STZ-mediated activation, because the maximal accumulation of 1,2-DG occurs rather late (>3 min) whereas the hydrolysis of PIP_2 is very fast and transient [17]. Moreover, we observed accumulation of inositol phosphates during activation with fMLP, but not during stimulation with STZ (not shown). The latter result indicates that even if STZ induces PIP_2 breakdown, the amount of 1,2-DG liberated from this hydrolysis is much less than with fMLP as activator (fig.1).

Most importantly, the present study shows that under various conditions there is no correlation between 1,2-DG accumulation and respiratory burst activation. The indications for this conclusion are as follows: (i) both PAF and fMLP give a small and comparable rise in 1,2-DG levels, but only fMLP is able to induce respiratory burst activity; (ii) the respiratory burst of neutrophils primed by PAF and activated by fMLP reaches a relative high activity without a concomitant rise in the level of 1,2-DG; (ii) during the first minute of the STZ-induced activation, the respiratory burst reaches 70% of its maximal activity without any rise in 1,2-DG levels (see figs 2,3).

A very recent report [18] evaluates the importance of 1,2-DG formation and activation of human neutrophils by Ca^{2+}-mobilizing ligands in the presence of cytochalasin B. Despite the fact that these authors used another methodology to measure 1,2-DG accumulation (short-term labeling of neutrophils with [^{3}H]arachidonate and [^{3}H]glycerol) and other conditions of activation, their study also, provides evidence for a lack of correlation between 1,2-DG formation and respiratory burst activation.

Under conditions of respiratory burst activity without accumulation of 1,2-DG (i.e. in the first period after STZ addition), the response is not sensitive to staurosporine. On the other hand, during the second phase of the STZ response, which is accompanied by a rise in the level of 1,2-DG, the respiratory burst becomes markedly sensitive to PK-C inhibition (see fig.5), suggesting a role for PK-C in the propagation of the oxidative response under these conditions. So far, the involvement of PK-C in the activation of the respiratory burst has been attributed to the phosphorylation of a 47 kDa protein [19,20]. The present results are in agreement with this hypothesis, because STZ-induced phosphorylation of this protein in neutrophil cytoplasts cannot be detected in the onset of the response (Kramer, IJ.M., Verhoeven, A.J. and Roos, D., unpublished), but is clearly present 5 min after stimulation [20]. The partial inhibition of the fMLP-induced response in PAF-primed cells by staurosporine suggests that this response is mediated by more than one activation mechanism.

Thus far, we have no indications as to which second messenger other than 1,2-DG is responsible for signal transduction between receptor occupation and NADPH oxidase activation. Interestingly, activation of the NADPH oxidase in a cell-free system requires, apart from SDS or arachidonate, the presence of GTP [21]. Therefore, it is possible that activation of the respiratory burst in intact neutrophils can also be achieved by a direct coupling between specific cell-surface receptors and membrane-bound components of the NADPH oxidase without the involvement of second messengers. Clearly, additional studies are required to substantiate this alternative model of respiratory burst activation.

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