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Platelet-activating factor (PAF) acts as an intercellular messenger in the changes of cytosolic free Ca^{2+} in human neutrophils induced by opsonized particles

Anton T.J. Tool, Arthur J. Verhoeven, Dirk Roos and Leo Koenderman

Central Laboratory of the Netherlands Red Cross, Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

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Additon of opsonized particles to human neutrophils in suspension leads to a biphasic elevation in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). The rise in $[Ca^{2+}]_i$ during the second phase (> 3 min) is pronounced (about 400 nM), in contrast to the rise during the first phase, which is relatively small (< 100 nM). The second and large rise in $[Ca^{2+}]_i$ is brought about by messenger(s) released from the cell after addition of opsonized particles. This second rise in $[Ca^{2+}]_i$ is not observed in the presence of the platelet-activating factor (PAF) antagonist WEB 2086, indicating that PAF can act as an intercellular messenger affecting Ca^{2+} homeostasis in human neutrophils.

$[Ca^{2+}]$, cytosolic free; Human neutrophil; Opsonized particle; Platelet-activating factor; Intercellular messenger

1. INTRODUCTION

Phagocytosis and killing of bacteria by neutrophils are important mechanisms in the host defence against invading microorganisms. The killing process is mediated by the concomitant release of cytotoxic proteins and oxygen metabolites into the phagolysosomes. The toxic oxygen metabolites are produced in a so-called 'respiratory burst' [1,2]. In vitro, these processes can be activated by the addition of opsonized particles, such as serum-treated zymosan (STZ, see e.g. [3]).

Not much is known about the second messengers that mediate the activation of human neutrophils after STZ stimulation. Several studies have shown that the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased upon STZ stimulation [4,5]. In many different cell types, changes in $[Ca^{2+}]_i$ are caused by the rapid hydrolysis of phosphatidylinositolbisphosphate (PIP_2) with the subsequent production of inositol-(1,4,5)trisphosphate (IP_3) and inositol-(1,3,4,5)tetrakisphosphate (IP_4) [6,7]. IP_3 has been shown to liberate Ca^{2+} ions from intracellular stores, whereas IP_4 has been implicated in the influx of Ca^{2+} ions from the extracellular medium into the cells [7]. Indeed, some studies have shown that activation of human neutrophils with opsonized particles is accompanied by the production of IP_3, although this response seems to be much slower than that caused by stimulation with chemoattractants [5,8]. These results have been interpreted as an indication that the opsonin receptors relevant for binding of STZ to neutrophils are coupled to a PIP_2-dependent phospholipase C. However, the possibility of intercellular messengers in this process has not been addressed.

In the present study, we have reinvestigated the Ca^{2+} response in human neutrophils after STZ stimulation. We have found that the rise in $[Ca^{2+}]i$ consists of two phases: a first phase with a relatively small increase in $[Ca^{2+}]i$ and a second phase with a much larger increase. This second phase is caused by the production of an intercellular messenger, most likely platelet-activating factor (PAF).

2. MATERIALS AND METHODS

2.1. *Materials*

STZ was prepared as described [9]. Indo-1/AM (Molecular Probes, Junction City, OR, USA) and WEB 2086 were dissolved in dimethyl sulfoxide (DMSO) at 1000 times the final concentration for cell incubations, and stored at −70°C. WEB 2086 was a kind gift from Dr H. Heuer (Boehringer Ingelheim, FRG). 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 serum 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 anticoagulated with 0.4% (w/v) trisodium citrate (pH 7.4), as described [10]. After isolation, the cells were suspended in incubation medium and kept at room temperature.

Correspondence address: J. Verhoeven, c/o Publication Secretariat, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, PO Box 9406, 1006 AK, Amsterdam, The Netherlands

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2.3. *Measurement of oxygen consumption*

Oxygen consumption was measured at 37°C with an oxygen electrode as described before [10].

2.4. *Measurement of cytosolic free calcium*

Cytosolic free Ca^+ was measured exactly as described before [11]. In short, prewarmed cells (5 min at 37°C, 25×10^6/ml of incubation medium) were incubated with 0.5 μM Indo-1/AM for 40 min at 37°C. Subsequently, the cells were washed, resuspended in incubation medium and kept at room temperature. Fluorescence measurements were performed at 37°C in stirred suspensions. Calibration of Indo-1 fluorescence as a function of $[Ca^{2+}]_i$ was determined as follows: to saturate all trapped Indo-1 with Ca^{2+}, 5 μM digitonin was added to the cell suspension. Subsequently, the Indo-1 signal was quenched by adding 0.5 μM Mn^{2+}. Cytosolic free Ca^{2+} was calculated as described [12], using 250 nM as the K_d for the Indo-1/Ca^{2+} complex [13].

3. RESULTS

3.1. *Measurement of cytosolic free Ca^{2+} after STZ stimulation*

Addition of STZ (0.1 mg/ml) to human neutrophils leads to a small increase in $[Ca^{2+}]_i$ in the first few minutes, as shown in fig.1. However, after 3.5 ± 0.4 min (mean ± SD, $n = 5$) a second rise in $[Ca^{2+}]_i$ occurs to values of 509 ± 114 nM (mean ± SD, $n = 5$). Part of this rise is caused by Ca^{2+} influx from the extracellular medium, as deduced by measurements of Mn^{2+} influx (see [14], data not shown).

Although the dose of STZ used in these experiments is suboptimal for activation of the respiratory burst, oxygen uptake increases rapidly up to 40–50% of its maximal value, as induced by the optimal concentration of STZ (1 mg/ml) (fig.2). For the Ca^{2+} measurements, higher doses of STZ were not applied, because of interference of the STZ particles with the fluorescence signal of Indo-1. A constant rate of oxygen uptake is reached within 3 min after addition of STZ. Thus, the second large rise in $[Ca^{2+}]_i$ after STZ addition occurs after activation of the respiratory burst has been completed.

We subsequently tried to determine whether the messenger for the second rise in $[Ca^{2+}]_i$ is acting intra- or intercellularly. For this purpose, the neutrophils were stimulated with STZ, and $[Ca^{2+}]_i$ was measured.

Fig.1. The effect of addition of opsonized particles (STZ) to human neutrophils on the $[Ca^{2+}]_i$. STZ (0.1 mg/ml) was added after 10 min preincubation of the cells (2.0×10^6/ml of incubation medium) at 37°C. The figure has been corrected for the change in basal fluorescence caused by the addition of the opsonized particles, as determined with unloaded cells. Results shown are representative for five different experiments.

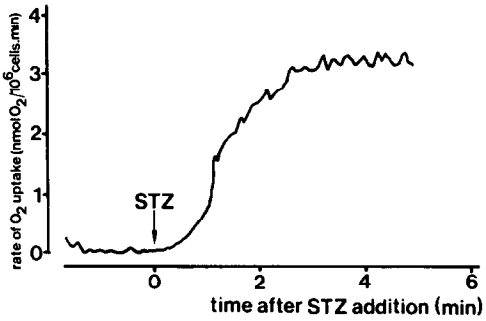

Fig.2. Computer evaluation of the time course of the rate of oxygen uptake by human neutrophils after activation with STZ. STZ (0.1 mg/ml) was added after a 10 min preincubation of the cells (2.0×10^6 /ml of incubation medium) at 37°C. Results shown are representative for five experiments.

After the second rise in cytosolic free Ca^{2+} had occurred (5 min after STZ addition), the cell suspension was centrifuged. Part of the supernatant was then added to unstimulated cells and $[Ca^{2+}]_i$ was measured. As shown in fig.3 (trace A), addition of this supernatant induced a fast and transient rise in the $[Ca^{2+}]_i$. The same experiment was carried out with the supernatant of cells that had been stimulated with STZ for only one minute. This supernatant did not result in changes in $(Ca^{2+})_i$ (fig.3., trace B). This experiment suggests that the second rise in $[Ca^{2+}]_i$ observed after stimulation of neutrophils with STZ is due to messengers acting intercellularly.

3.2. *Effect of the PAF-antagonist WEB 2086 on the changes in $[Ca^{2+}]_i$*

After activation of neutrophils with STZ, two lipid mediators are produced which at low concentrations are able to elevate $[Ca^{2+}]_i$: PAF and leukotriene B4 (LTB4) [15,16]. To investigate the possibility that one of these mediators was involved in the $[Ca^{2+}]_i$ changes observed

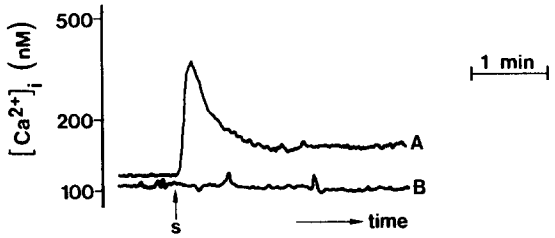

Fig.3. The effect of the addition of supernatant of human neutrophils that were stimulated with STZ on the $[Ca^{2+}]_i$ of human neutrophils. STZ (0.1 mg/ml) was added to Indo-1-loaded cells and $[Ca^{2+}]_i$ was measured. After the second rise in $[Ca^{2+}]_i$ had occurred (5 min after STZ addition), the cell suspension was centrifuged. Part of the supernatant (s) was then added to unstimulated cells and $[Ca^{2+}]_i$ was measured (trace A). The control experiment was performed with supernatant of cells that were stimulated with STZ for only one minute (trace B). The figure has been corrected for the change in basal fluorescence caused by the addition of supernatant (1 ml) to the cell suspension (2 ml), as determined with addition of buffer to the cell suspension. Results shown are representative for five experiments.

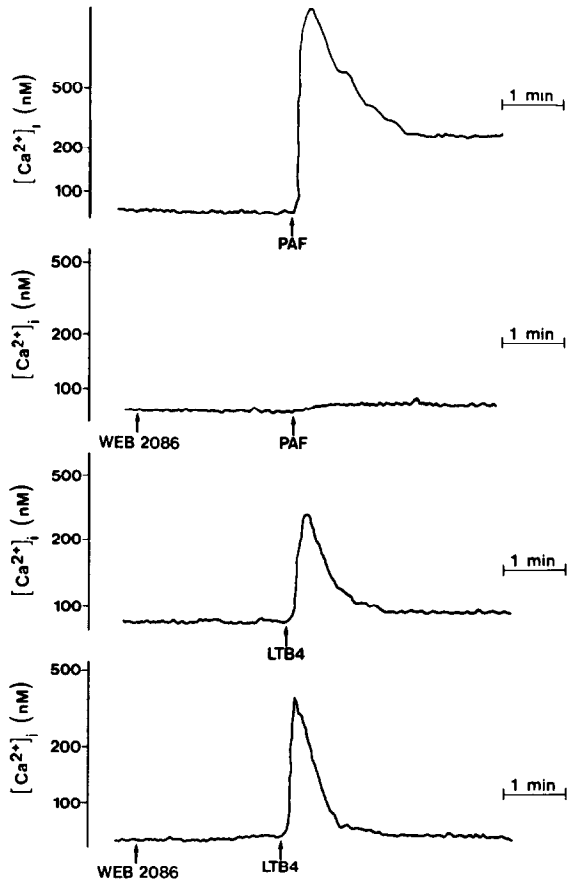

Fig.4. The effect of the PAF antagonist WEB 2086 on the change in $[Ca^{2+}]_i$ induced by PAF and LTB4. WEB 2086 (10 μM) or buffer were added 2 min prior to PAF (1 nM) or LTB4 (1 nM). The results shown are representative for five experiments.

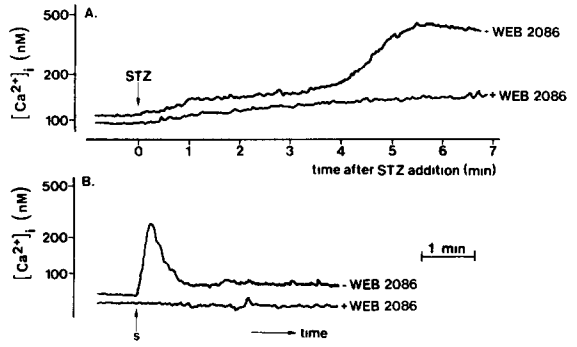

Fig.5. The effect of the PAF antagonist WEB 2086 on the changes in $[Ca^{2+}]_i$ induced by STZ (A) and by supernatant of neutrophils that were stimulated with STZ for 5 min (B). WEB 2086 (10 μM) or buffer were added 2 min prior to STZ (0.1 mg/ml, A) or 2 min prior to the supernatant (s) of STZ (0.1 mg/ml) stimulated neutrophils (B). (A) has been corrected for the change in basal fluorescence induced by the addition of the STZ particles, as determined with unloaded cells. (B) has been corrected for the change in basal fluorescence induced by the addition of supernatant (1 ml) to the cell suspension (2 ml), as determined by addition of buffer to the cell suspension. Results shown are representative for five experiments.

with STZ, we first investigated the sensitivity of these changes for the PAF-antagonist WEB 2086 [17]. WEB 2086 (10 μM) completely inhibited the increase in $[Ca^{2+}]_i$ induced by 1 nM PAF, while the rise in $[Ca^{2+}]_i$ induced by 1 nM LTB4 was not affected (fig.4). WEB 2086 had no influence on the respiratory burst of human neutrophils stimulated with STZ (data not shown).

Preincubation of neutrophils with 10 μM WEB 2086 did not affect the small increase in $[Ca^{2+}]_i$ in the first minutes after STZ stimulation (fig.5A). However, the second rise in cytosolic free Ca^{2+} was completely inhibited by 10 μM WEB 2086. This indicates that the second rise in $[Ca^{2+}]_i$ is caused by the release of PAF, which subsequently acts as an intercellular messenger. To strengthen this hypothesis, we investigated the effect of WEB 2086 on the rise in $[Ca^{2+}]_i$ induced by the supernatant of STZ-treated neutrophils. As shown in fig.5B, this increase in $[Ca^{2+}]_i$ was completely absent when the neutrophils were preincubated with 10 μM WEB 2086 before addition of the supernatant.

4. DISCUSSION

In several studies it has been found that activation of human neutrophils with opsonized particles is accompanied by a rise in $[Ca^{2+}]_i$ [4,5] and hydrolysis of PIP_2 with the subsequent production of inositolphosphates [5,8]. The predominant opsonin on STZ particles is iC3b [18,19]. Although these particles also carry IgG [3], the binding of this opsonin to Fc-gamma receptors on the neutrophil is prevented by steric hindrance by the iC3b bound to the Fc region of IgG [3,19]. Thus, it could be argued that the iC3b receptor is mediating this second messenger response via direct activation of phospholipase C. However, when STZ is added at a concentration that is sufficient to induce 40–50% of the maximal respiratory burst activity, only a small rise in $[Ca^{2+}]_i$ occurs following receptor occupation. We were not able to detect increases in inositolphosphates during the first 2–3 min after STZ stimulation (data not shown). In another study, we have shown that accumulation of diglycerides occurs only after prolonged (>90 s) stimulation with STZ [20]. So, if a direct coupling between the iC3b receptor and a PIP_2-dependent phospolipase C is present, the rise in IP_3, IP_4 and $[Ca^{2+}]_i$ resulting from this coupling is only a minor event under our experimental conditions. On the other hand, a second and much larger rise in $[Ca^{2+}]_i$ occurred after STZ addition, mediated by an intercellular messenger. The potent inhibition of this increase in $[Ca^{2+}]_i$ by the PAF antagonist WEB 2086 strongly suggests that this intercellular messenger is identical with PAF. Addition of supernatant of neutrophils activated with STZ induces a transient rise in the $[Ca^{2+}]_i$, in analogy to activation with a single dose of PAF (see fig.4). The finding that the second rise in $[Ca^{2+}]_i$ after

STZ stimulation is a sustained rise, is presumably caused by the constant production of PAF by the activated neutrophils. Although our data clearly indicate that the second rise in $[Ca^{2+}]_i$ does not affect the respiratory burst, this response may be important for the fusion of granules with the phagosomes formed [21].

Upon activation of neutrophils with STZ, PAF is rapidly produced from (plasmalogen) phosphatidylcholine via activation of phospholipase A_2 (PLA_2) and acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine-O^2-acetyltransferase, succesively [22,23]. Our findings indicate that PAF is synthesized while cytosolic free Ca^{2+} increases only very little (less than 100 nM). Presumably, another signal than cytosolic free Ca^{2+} is required for the activation of PLA_2. The requirement of PLA_2 for Ca^{2+} could be lowered by the involvement of G-proteins [24]. Interestingly, acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine-O^2-acetyltransferase, which is the rate-limiting step in the synthesis of PAF, seems to be controlled by a phosphorylation/dephosphorylation mechanism without requirement of Ca^{2+} [22].

In summary, our study indicates that a major part of the Ca^{2+} response of neutrophils after stimulation with STZ particles is caused by the production of PAF by the stimulated cells. Further studies are required to establish the mechanism by means of which this autocrine response in neutrophils is activated.

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