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Adherence of human neutrophils changes Ca^{2+} signaling during activation with opsonized particles

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Changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) upon activation of human neutrophils by opsonized particles (serum-treated zymosan; STZ) were evaluated by three different methods: (i) measurement of total fluorescence changes in indo-1 loaded neutrophils activated in suspension; (ii) measurement of fluorescence changes in individual indo-1 loaded neutrophils in a flow cytometer and (iii) measurement of fluorescence changes in individual fura-2 loaded neutrophils adherent to serum-coated coverslips. Our study shows that the opsonized particle-induced change in $[Ca^{2+}]_i$ in neutrophils is altered during adherence of the cells to a serum-coated surface. These observations might be of importance for neutrophil function in vivo, since adherence is a prerequisite for diapedesis and chemotaxis.

Neutrophil; Adherence; Ca^{2+} signaling

1. INTRODUCTION

Human neutrophils are important effector cells in the host defense against invading microorganisms. After contact with neutrophils, the opsonized microorganisms are phagocytized and killed intracellularly by the release of preformed cytotoxic proteins from granules into the phagolysosome [1] and the generation of toxic oxygen metabolites by a membrane-bound NADPH oxidase, referred to as the respiratory burst [2]. Accompanying neutrophil activation is the production of newly synthesized lipid mediators [3,4]. In vitro, these processes can be activated by the addition of opsonized particles, such as serum-treated zymosan (STZ), or by high doses of chemoattractants [5,6].

It is well established now that activation of human neutrophils with chemoattractants is accompanied by a G-protein-mediated activation of phospholipase C, resulting in a rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate and 1,2-diacylglycerol [7–10]. Recent studies have indicated that the phosphoinositol cycle is activated during stimulation of human neutrophils with opsonized particles, and that addition of opsonized particles induces elevations in $[Ca^{2+}]_i$ [11–13]. This has been interpreted as an indication that opsonin receptors are directly coupled to a PIP_2-hydrolysing phospholipase C. However, we recently demonstrated that the major part of the elevated $[Ca^{2+}]_i$ in phagocytizing neutrophils was caused by the intercellular release of PAF by the activated neutrophils [14]. However, in these measurements of total fluorescence changes in suspended neutrophils, small and asynchronous oscillations in $[Ca^{2+}]_i$ induced by the particles might have remained undetectable. To eliminate this possibility we applied single-cell analysis by flow cytometry [15]. With this technique we demonstrate that phagocytosis of opsonized particles by neutrophils in suspension is not accompanied by changes in $[Ca^{2+}]_i$. In contrast, adherent neutrophils do respond with a rapid rise in $[Ca^{2+}]_i$.

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Abbreviations: STZ, serum-treated zymosan; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; fMLP, formyl-methionyl-leucyl-phenylalanine; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PK-C, protein kinase C

2. MATERIALS AND METHODS

2.1. *Materials*

Formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma Chemical Co. St. Louis, MO, USA), digitonin (Merck, Darmstadt, FRG) and indo-1/AM (Molecular Probes, Junction City, OR, USA) were dissolved in DMSO at 1000 times the final concentration for cell incubations, and were stored at −20°C. Serum-treated zymosan (STZ) was prepared as described before [16]. All other chemicals were reagent grade. Incubation medium consisted of 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 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. *Neutrophil isolation*

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

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13 mM trisodium citrate (pH 7.4). The buffy coat was diluted three times with phosphate-buffered saline containing 13 mM trisodium citrate, and centrifuged (20 min, 1000×g, room temperature) over Percoll with a specific gravity of 1.077 g/cm^3. The erythrocytes in the pellet fraction were lysed with isotonic ammonium chloride at 4°C, as described elsewhere [5]. The neutrophils were washed and resuspended in incubation medium, and were kept at room temperature until use.

2.3. *Measurement of $[Ca^{2+}]_i$*

2.3.1. Measurement of $[Ca^{2+}]_i$ in neutrophils in suspension

The neutrophils (2.5×10^7 cells/ml) were loaded with indo-1 by incubating the cells with 1 μM of the acetoxy-methyl ester indo-1/AM [17] for 40 min at 37°C. The cells were then washed, resuspended in incubation medium and kept at room temperature until use. Before each measurement, the neutrophils were diluted in incubation medium (final concentration 10^6 cells/ml) and incubated at 37°C for 15 min before transfer to a cuvette. Fluorescence measurements were performed with a spectrofluorometer (model RF-540; Shimadzu Corporation, Kyoto, Japan) exactly as described before [5].

2.3.2. Measurement of $[Ca^{2+}]_i$ in neutrophils in suspension at the single cell level

The $[Ca^{2+}]_i$ in individual neutrophils in suspension was measured by a flow cytometer. The flow-cytometric analysis was performed with a FACSTAR-PLUS (Becton Dickinson, Mountain View, CA, USA). Indo-1 was excited by 100 mW, 351–364 nm illumination from an argon-ion laser (Coherent 90/5 UV, Palo Alto, CA, USA). Free indo-1 emission was measured at 463–507 nm (DF 485/22 nm, Becton Dickinson) and Ca^{2+}/indo-1 emission at 385–425 nm (DF 405/20 nm, Becton Dickinson). The emission signals were separated by means of a 505 nm SP filter placed under an angle of 45°. Prewarmed neutrophils loaded with indo-1 (see section 2.3.1.) were transferred to a magnetically stirred delivery system, which was kept at 37°C. The cells were continuously analyzed at a rate of 100 cells/s. The indo-1 emission ratio as a function of $[Ca^{2+}]_i$ was calculated from 50000 cells, using the kinetic software INCA (developed by J.F. Key, TNO, Rijswijk, The Netherlands).

2.3.3. Measurement of $[Ca^{2+}]_i$ in single adherent neutrophils

The neutrophils (2.5×10^7 cells/ml) were loaded with fura-2 by incubation with 5 μM fura-2/AM for 60 min at 37°C. After the loading procedure, the cells were washed and kept at room temperature until use.

Coverslips were coated with autologous serum by incubation for 30 min at 37°C. The coverslips were then washed, and fura-2-loaded neutrophils were placed on the coated coverslip and incubated for 15 min at 37°C. Non-adherent cells were removed by washing, and the coverslip with adherent neutrophils was placed in a Leidem Cell Chamber maintained at 37°C (Medical Systems, Greenvale, NY, USA). The fluorescence of the fura-2 loaded cell was imaged with a 100× Nikon Fluor objective and a Nikon Diaphot epifluorescence microscope illuminated with alternating (100 Hz) 340 and 380 nm (5 nm HBW) light. Fluorescent emission was collected at 520 nm (50 nm HBW) by a photomultiplier tube connected to a Johnson Foundation Spinning Wheel Fluorimeter (University of Pennsylvania Biomedical Instrumentation Group, Philadelphia, PA, USA). Output from the fluorimeter was digitized and analyzed by an IBM AT clone based analysis system (Indec Systems, Sunnyvale, CA, USA). Calibration of the fluorescence signal was performed in situ by addition of 1 μM ionomycin (R_{max}), followed by addition of 5 mM EGTA, pH 8.0 (R_{min}). Values obtained were 0.5, 3.5 and 7.1 for R_{min}, R_{max} and B, respectively. A K_d of 224 nM for the Ca^{2+}/fura-2 complex was used [17].

Opsonized particles (STZ particles) were directly brought into contact with the neutrophil surface by picking them up with the micropipette tip and moving them to the cell. fMLP was delivered to individual cells via a micropipette positioned approximately 20 μm from the cell surface via a Newport micromanipulator. The content of the micropipette was applied to the cell surface by delivery of a pulse of nitrogen gas (3–5 psi) for various durations to the micropipette.

3. RESULTS

When opsonized particles (0.1 mg/ml) were added to human neutrophils in suspension (2×10^6 cells/ml), a small rise in $[Ca^{2+}]_i$ was detected during the first 3–4 min (see Fig. 1A). After 4 min a large and more rapid rise in $[Ca^{2+}]_i$ occurred, which was caused by the release and intercellular action of platelet-activating factor [14]. In contrast, the neutrophils incubated under the same conditions did respond rapidly with a change in $[Ca^{2+}]_i$ after addition of the chemoattractant fMLP (Fig. 1B).

We subsequently measured changes in $[Ca^{2+}]_i$ in a neutrophil suspension at the single-cell level in a flow cytometer. As is shown in Fig. 2A, no dectectable increase in $[Ca^{2+}]_i$ was noticed during the first few minutes after addition of the particles, while phagocytosis occurred as deduced from microscopic inspection. After 3–5 min, a small rise in $[Ca^{2+}]_i$ is present, that can probably be attributed to the release of PAF. Under the same conditions, the neutrophils were responsive to fMLP (Fig. 2B). This indicates that phagocytosis of opsonized particles is not necessarily accompanied by increases in $[Ca^{2+}]_i$. Under the same conditions the respiratory burst is optimally activated within 3–4 min [14].

Our findings may seem in conflict with data from other investigators, who have demonstrated an increase

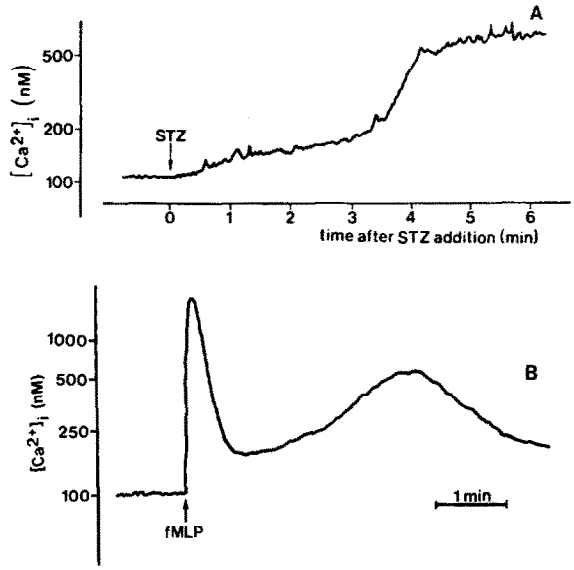

Fig. 1. The effect of addition of opsonized particles (STZ) or the chemoattractant fMLP on $[Ca^{2+}]_i$ of human neutrophils in suspension. After 15 min preincubation at 37°C, indo-1 loaded neutrophils were transferred to a thermostatted (37°C) and stirred cuvette, and STZ (0.1 mg/ml) (A) or fMLP (100 nM) (B) was added at the indicated time. The change in total fluorescence is expressed and reflects the mean change of the $[Ca^{2+}]_i$ in the individual cells. The result shown is representative for 10 different experiments.

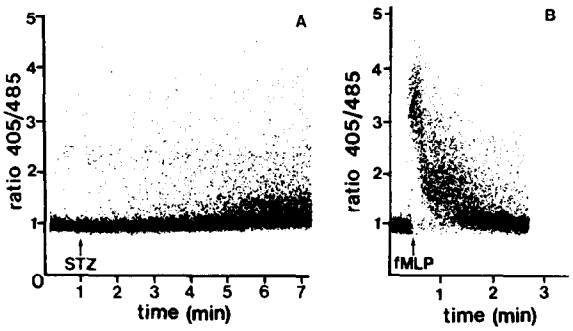

Fig. 2. The effect of addition of opsonized particles (STZ) or fMLP on $[Ca^{2+}]_i$ of human neutrophils in suspension measured at the single-cell level in a flow cytometer. After 15 min preincubation at 37°C, indo-1 loaded neutrophils were transferred to a thermostatted (37°C) and stirred tube. STZ (0.1 mg/ml) (A) or fMLP (100 nM) (B) was added at the indicated time. The ratio of the excitation fluorescence signals at 405 nm and 485 nm (a measure of $[Ca^{2+}]_i$) was assessed in the individual cells at a rate of 100 cells/s. The result shown is representative for 5 different experiments.

in $[Ca^{2+}]_i$ during phagocytosis of opsonized particles [11,18]. However, these observations were made in adherent neutrophils. Therefore, we repeated the experiments by measuring $[Ca^{2+}]_i$ of single, adherent neutrophils (see Materials and Methods for details).

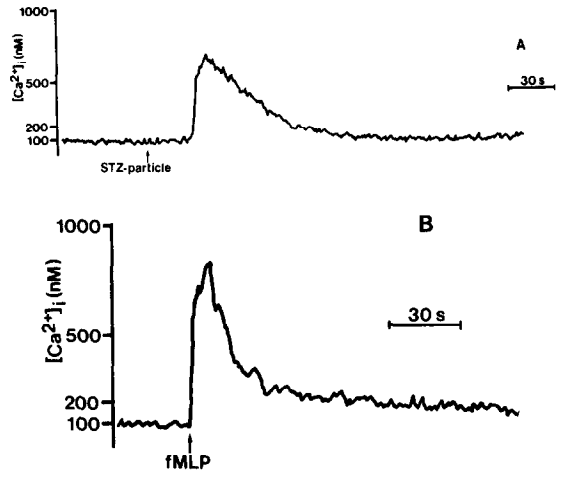

Fig. 3. The change in $[Ca^{2+}]_i$ in a single adherent neutrophil during phagocytosis of an STZ particle or after activation with fMLP. Fura-2 loaded neutrophils were placed on serum-coated coverslips (for experimental details see Materials and Methods). After 15 min at 37°C, the non-adherent neutrophils were removed by washing, and the coverslip was transferred to a thermostatted (37°C) chamber. (A) An STZ particle was brought into contact with a neutrophil at the time indicated with an arrow, and $[Ca^{2+}]_i$ was measured. During the latency period and after the rise in $[Ca^{2+}]_i$, visual inspection was made for identification of phagocytosis. (B) A short pulse (1 s, 5 psi) of fMLP (100 nM) was delivered to an individual cell via a micropipette positioned approximately 20 μm from the cell surface. By this procedure, the cell is stimulated for a very short time with 100 nM fMLP. Hereafter, the concentration of fMLP rapidly decreases by dilution of the chemoattractant in the extracellular medium. The experiment shown is representative for three different experiments performed in triplicate.

After contact between a neutrophil and an STZ particle was established (as deduced by visual inspection), a latency period of about 10–30 s was present without a rise in $[Ca^{2+}]_i$ (see Fig. 3A). During this period no phagocytosis was observed. After this period a rapid and large rise in $[Ca^{2+}]_i$ occurred. $[Ca^{2+}]_i$ declined again to base line level within 1 min. Visual inspection was made for positive identification of phagocytosis. Under the same conditions, adherent neutrophils were responsive towards fMLP (Fig. 3B).

4. DISCUSSION

Signal transduction between receptor activation and neutrophil activation has been the subject of many recent studies (for review see [19]). Most studies have been performed with neutrophils in suspension. However, recent studies have demonstrated that neutrophils adhering to biological surfaces show different characteristics of activation compared with neutrophils in suspension [20–22].

The present study was designed to compare Ca^{2+} signaling in granulocytes adherent to a biological surface with neutrophils in suspension. With respect to activation with opsonized particles, several studies seem in accord with each other. Mandell and co-workers have shown that $[Ca^{2+}]_i$ is increased during phagocytosis of opsonized particles by adherent neutrophils [11,18]. This finding was confirmed with neutrophils in suspension, although the kinetics of the response were not comparable [12]. In addition, PIP_2 hydrolysis during phagocytosis of opsonized particles by neutrophils in suspension was shown by measuring inositol phosphates and DAG [12,13]. Taken together, these studies suggested that PLC activation results from the binding of opsonized particles to opsonin receptors on neutrophils.

Studies in our laboratory have failed to confirm this conclusion [14,23]. Moreover, other investigators have shown that phagocytosis can occur in the absence of changes in $[Ca^{2+}]_i$ [24]. Therefore, we re-evaluated the changes in $[Ca^{2+}]_i$ after addition of opsonized particles. We have now measured the $[Ca^{2+}]_i$ at the single cell level in both adherent cells and cells in suspension. When an STZ particle was brought into contact with an adherent neutrophil, a rise in $[Ca^{2+}]_i$ occurred during phagocytosis of this particle (see Fig. 3), thereby confirming earlier studies. In sharp contrast, addition of STZ to neutrophils in suspension did not lead to an increase in $[Ca^{2+}]_i$ as measured at the single cell level in a flow cytometer (see Fig. 2). Although with this technique the response of a single neutrophil cannot be followed in time, it provides an extensive statistical analysis of the events occurring in a neutrophil suspension. Asynchronous changes in the cells upon addition of opsonized particles would have resulted in a broadening of the signals detected in the flow cytometer. The absence of

changes in $[Ca^{2+}]_i$ in the neutrophils in suspension cannot be explained by the use of indo-1 (necessary for flow cytometric analysis) instead of fura-2, because both gave identical results when neutrophils in suspension were tested in the spectrofluorimeter (results not shown). Apparently, the adhesion event causes a significant change in the Ca^{2+} signaling properties of the opsonin receptors. It should be noted, however, that the fMLP-induced changes in $[Ca^{2+}]_i$ are unchanged after adhesion.

A striking feature of animal cells, which has recently been discovered by studying adherent cells at the single cell level, is the presence of $[Ca^{2+}]_i$ oscillations [25]. These oscillations have also been described in neutrophils [26]. During our studies on single adherent neutrophils we found only few neutrophils in which $[Ca^{2+}]_i$ oscillations occurred (not shown). It is not clear at present which circumstances determine the number of oscillating neutrophils.

In conclusion, our study indicates that after adherence of human neutrophils the transmembrane signaling during phagocytosis is changed. It is not yet known which mechanism is responsible for this change. It is tempting to speculate on an association of a PIP_2-hydrolyzing PLC with the opsonin receptors during adherence.

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