On the mechanism of oscillations in neutrophils

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ABSTRACT

We have investigated the regulation of the oscillatory generation of H_2O_2 and oscillations in shape and size in neutrophils in suspension. The oscillations are independent of cell density and hence do not represent a collective phenomena. Furthermore, the oscillations are independent of the external glucose concentration and the oscillations in H_2O_2 production are 180° out of phase with the oscillations in NAD(P)H. Cytochalasin B blocked the oscillations in shape and size whereas it increased the period of the oscillations in H_2O_2 production. 1- and 2-butanol also blocked the oscillations in shape and size, but only 1-butanol inhibited the oscillations in H_2O_2 production. We conjecture that the oscillations are likely to be due to feedback regulations in the signal transduction cascade involving phosphoinositide 3-kinases (PI3K). We have tested this using a simple mathematical model, which explains most of our experimental observations.

1. Introduction

Neutrophilic granulocytes are a part of the innate immune system and they are especially involved in clearance of bacterial and fungal infections. Neutrophils will usually phagocytize and degrade micro-organisms internalized in the phagosomes [1]. An important component of the phagosomal membrane is the transmembrane enzyme complex NADPH oxidase that oxidizes NADPH in the cytosol and reduces oxygen to superoxide on the other side of the membrane. It consists of two membrane bound components (gp91[PHOX] and p22[PHOX]), three cytosolic units (p67[PHOX], p47[PHOX] and p40[PHOX]) and an additional regulatory cytosolic component Rac. Activation of NADPH oxidase involves phosphorylation of p47[PHOX] by protein kinases, e.g. protein kinase C (PKC), and translocation of the cytosolic subunits to the membrane. During activity of NADPH oxidase, p47[PHOX] is continuously dephosphorylated and re-phosphorylated, and lack of re-phosphorylation inactivates the oxidase [2,3]. Upon stimulation of the neutrophil NADPH oxidase is assembled and activated in the plasma membrane and/or the phagosome membrane [2]. NADPH oxidase can be activated in vitro with the formylated peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) or the protein kinase C activator phorbol-12-myristate-13-acetate (PMA) [2,3]. NADPH oxidase depolarizes the membrane it is spanning due to the electrogenic properties of the reaction it catalyzes [3,4].

Superoxide formed by the NADPH oxidase reaction dismutates spontaneously into oxygen and H_2O_2 [3,4] which can easily be detected with good time resolution. When neutrophils in suspension are stimulated with both PMA and fMLP following incubation with wortmannin they show damped oscillations in the production of H_2O_2 together with apparently synchronized oscillations in shape or size, but the oscillations can also be observed if wortmannin is omitted [5,6]. The oscillations in morphology and production of H_2O_2 are accompanied by an increase in intracellular Ca^{2+}. However, Ca^{2+} oscillations have not been observed under these conditions [5,7]. The mechanism responsible for the oscillations in morphology and respiratory burst remains unknown. Sustained oscillations and wave patterns in NAD(P)H, H_2O_2 and Ca^{2+} inside single adhered neutrophils have been reported by Petty et al. [8–10]. The period of the oscillations changes rapidly following stimulation with fMLP or glucose and these oscillations are speculated to be similar to the damped oscillations in a suspension of neutrophils. However, no other groups have, to the best of our knowledge, reported similar observations and doubts have recently been raised [11,12] about the observations published by Petty and co-workers.

To explore the nature of the oscillations and the biochemical mechanism behind them, we have investigated the pulsatory H_2O_2 production in neutrophils stimulated with PMA and fMLP. We have also measured NAD(P)H auto-fluorescence and observed damped oscillations in NAD(P)H, with a period and waveform that correspond to the oscillations in H_2O_2 production. We measured the oscillations in H_2O_2 generation at different cell densities, and found that the oscillations are independent of the cell density. This indicates that, as opposed to yeast cells which undergo an oscillating glycolysis [13,14], the oscillations in individual neutrophil do not depend on the exchange of one or more specific synchronizing compounds between cells. To address the biochemical mechanisms we incubated the

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neutrophils with the phospholipase D (PLD) inhibitor 1-butanol, which resulted in strong inhibition of the oscillations. Furthermore, addition of cytochalasin B, which completely blocks actin polymerization and hence also changes in shape and size of neutrophils, only affected the period of the oscillations in H_2O_2 production. To support and explain our experimental observations we constructed a mechanistic model of the positive feedback loop between phosphoinositide 3-kinases (PI3K) and Rac and with this model we can reproduce and explain most of the experimental observations qualitatively.

2. Experimental procedures

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (Munich, Germany), except for horseradish peroxidase (HRP) (Roche, Mannheim, Germany), Lymphoprep (Nycomed, Norway), 1-Butanol (Merck) and 2-Butanol (Fluka), Wright-Giemsa assay (Pharmacy, Odense University Hospital), and the fluorescent probes Amplex Red, 3,3′-dipentyloxacarbocyanine iodide (DiOC$_5$(3)), bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC$_4$(3)) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) that were obtained from Molecular Probes (Eugene, OR).

The HEPES buffer used for final re-suspension of neutrophils was made in house (130 mM NaCl, 5.0 mM NaHCO$_3$, 4.6 mM KC1, 1 mM CaC1$_2$, buffered with 20 mM HEPES and 1.1 mM KH$_2$PO$_4$, adjusted to pH 7.4 with 1 M NaOH). Glucose was later added to the buffer at final concentration used in the current experiment.

2.2. Neutrophil isolations

Neutrophils were isolated from a number of different donors using the same procedure as Theilgaard-Mönch et al. [15]. In short, 8 to 10 ml of peripheral blood (PB) was withdrawn from healthy donors giving informed consent. Blood was anticoagulated by sodium heparin and handled in plastic tubes. PB was depleted of erythrocytes using dextran sedimentation (2% Dextran T-500 in 0.9% NaCl). The supernatant was laid on Lymphoprep. Following centrifugation, the supernatant and the interphase were removed and the pellet was re-suspended in millipore water for lysis of remaining erythrocytes. After 30 s, an equal amount of 1.8% NaCl was added to stop lysis. The cells were washed twice using 0.9% NaCl and re-suspended in the final buffer to a final density of 10^6 cells/ml unless otherwise indicated. Following this step all cells were stored on ice.

All the experiments were performed at least three times with cells from different donors.

2.3. H_2O_2 measurements using luminol

H_2O_2 was measured using luminol as described by Wymann et al. [5]. Neutrophils were incubated at 37 °C with 10 μM luminol, 0.1 μM NaN$_3$ and 9 μg/ml HRP together with 1 μM wortmannin 10 min before the onset of the respiratory burst and then added to a stirred 2 ml sample in a 1 cm×1 cm×4.5 cm quartz cuvette which was maintained at 37 °C. The neutrophils were then stimulated with 3.25 nM PMA and after 3 min with 100 nM fMLP. The chemiluminescence was measured using a Hamamatsu R7400U-03 detector (PMT) that was mounted in an Edinburgh FS910 Spectrofluorometer (Edinburgh Instruments, Edinburgh, Scotland). For these measurements the spectrometer was operated in dark-mode, i.e. with the lamp switched off.

2.4. Fluorescence measurements

Measurements of NAD(P)H auto-fluorescence, Amplex Red, DiOC$_5$(3), DiBAC$_4$(3) and JC-1 fluorescence were measured in an Edinburgh FS910 Spectrofluorometer (Edinburgh Instruments, Edinburgh, Scotland) fitted with a temperature-controlled cuvette holder. Temperature was 37 °C.

2.4.1. NAD(P)H measurements

All NAD(P)H measurements were made in suspensions with 5×10^6 cells/ml in HEPES buffer with 5 mM glucose. NAD(P)H was excited at 366/3 nm (i.e. the wavelength was 366 nm with a deviation of 3 nm) and emission measured at 450/20 nm.

2.5. Light scattering

Changes in cell size/shape were measured as light scattering using a 636.2 nm picosecond pulsed diode laser (Edinburgh Instruments, Edinburgh, Scotland), (1 μs between pulses) mounted on an Edinburgh FS910 Spectrofluorometer (Edinburgh Instruments, Edinburgh, Scotland) fitted with a temperature-controlled cuvette holder. Temperature was 37 °C. For these measurements the spectrometer was operated in dark-mode, i.e. with the lamp switched off.

2.6. Membrane potentials

Membrane potentials were measured using DiOC$_5$(3), DiBAC$_4$(3) or JC-1. The neutrophils were incubated with 0.1 μM DiOC$_5$(3) [16] for 5 min at 37 °C prior to measurements and then transferred to the cuvette. When the signal was stable, the cells were stimulated with 100 nM PMA or 1 μM fMLP. Later, 10 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added to ensure that the cells were responding to the proton gradient uncoupler. DiOC$_5$(3) fluorescence was measured at excitation 486/3 nm and emission 510/5 nm [16–18]. Neutrophils were incubated with 0.1 μM JC-1 using the same procedure as with DiOC$_5$(3). Formation of JC-1 aggregates inside organelles was detected by measuring fluorescence at excitation 535/3 nm, emission 590/5 nm [19–21]. DiBAC$_4$(3) was added directly to the neutrophils after they had been transferred to the quartz cuvette. DiBAC$_4$(3) was measured with excitation at 493/3 nm and emission at 516/5 nm.

2.7. H_2O_2 estimation by Amplex Red

The neutrophils were incubated for 10 min at 37 °C with 50 μM Amplex Red, 1 mM NaN$_3$, 5.24 μg/ml HRP together with 1 μM wortmannin and transferred to a stirred quartz cuvette. 10 min after wortmannin addition, the cells were stimulated with 3.25 nM PMA and after further 3 min with 100 nM fMLP. The fluorescent product resorufin was detected by fluorescence with excitation 530/3 nm and emission at 590/3 nm.

2.8. Ca^{2+} measurements

The neutrophils were incubated with 2 μM Fura2/AM at 37 °C for 30 min, with 2 μM Fluo4/AM for 40 min, or with 1 μM Fluo5-F/AM for 30 min. After incubation, the cells were washed twice and re-suspended in HEPES buffer to a density of 10^6 cells/ml. Following this, the cells were incubated with 1 μM wortmannin 10 min prior to stimulation with 3.25 nM PMA and 3 min later 100 nM fMLP was added. Ca^{2+} binding to Fura2 was measured using excitation at 340/3 nm and emission at 500/10 nm. Ca^{2+} binding to Fluo4 was measured with excitation at 488/3 nm and emission at 516/10 nm, while binding to Fluo5-F was measured with excitation at 494/3 nm and emission at 518/10 nm.