Biophysical Chemistry 148 (2010) 82–92

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

On the mechanism of oscillations in neutrophils

Jens Christian Brasen [a,*], Torben Barington [b], Lars Folke Olsen [a]

[a] CelCom, Institute of Biochemistry and Molecular Biology, University of Southern Denmark, Denmark
[b] Department of Clinical Immunology, Odense University Hospital and Clinical Institute, University of Southern Denmark, Denmark

ARTICLE INFO

Article history:
Received 21 December 2009
Received in revised form 19 February 2010
Accepted 22 February 2010
Available online 25 February 2010

Keywords:
Neutrophil
Oscillations
Protein kinase C
NAD(P)H
NADPH oxidase

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 microorganisms 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

* Corresponding author. Tel.: +45 6550 2479; fax: +45 6550 2467.
E-mail address: christianb@bmb.sdu.dk (J.C. Brasen).

0301-4622/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2010.02.013

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_2PO_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 at 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.

2.9. Mathematical model

The model (Tables 1 and 2) was implemented in the software Berkeley-Madonna (University of California at Berkeley, Berkeley, CA) and Copasi (EML Research, Heidelberg, Germany and VBI, Blacksburg, VA, http://www.copasi.org) [22]. The numerical routine for integration was the Rosenbrock algorithm with maximum and minimum step sizes of 0.1 and 10^{-16}, respectively, and an absolute error of 10^{-15}. We repeated the simulations with the LDODA algorithm with relative and absolute errors of 10^{-6} and 10^{-12}, respectively. With the Madonna software the model was also simulated using a 4th order Runge–Kutta method (step size 10^{-6}), which gave identical results to those obtained with the Rosenbrock algorithm.

3. Results

3.1. Hydrogen peroxide oscillations

It has previously been shown that oscillations in shape and H_2O_2 production can be induced by treating neutrophils with the phosphoinositide 3-kinase inhibitor wortmannin followed by stimulation with PMA and fMLP [5,23]. While under some conditions oscillations may be observed in the absence of wortmannin and PMA [5,23], we have found that wortmannin and PMA gave the most reproducible conditions with the best signal. Fig. 1A shows the production of H_2O_2 in cells incubated with wortmannin and stimulated first with PMA and then with fMLP. Following PMA addition there is a delay of around 30–60 s before H_2O_2 production is at its maximum, as observed previously [5,24]. Fig. 1B is a magnification of the oscillatory regime in Fig. 1A. As judged from the plot the period is 7.4 ± 0.4 s, as reported previously [5], and matches observations of oscillatory changes in cell size and form [5,23]. When either wortmannin, PMA or fMLP was omitted, we did not observe any oscillations. However, if wortmannin was omitted we were still able to induce oscillations in H_2O_2 production when we stimulated the cells with 1.6 nM PMA and 10 nM fMLP (Fig. 2A).

The oscillations in H_2O_2 production have to the best of our knowledge only been measured with luminol together with HRP. However, oscillations have been observed in a number of HRP catalyzed reactions [25–27], including the oxidation of dihydroxyfumaric acid by molecular oxygen in the presence of luminol (L.F. Olsen, unpublished data). In order to exclude one possibility that the oscillations in H_2O_2 production were caused by an HRP catalyzed reaction involving luminol and that the neutrophils were otherwise affected by luminol [28], we have also used Amplex Red to assay H_2O_2 production. When Amplex Red is oxidized it becomes fluorescent and therefore the signal accumulates. In Fig. 2B we have estimated the derivation (slope) of the Amplex Red signal using a three point method ($(x_{t+h} - x_{t-h})/2h$). The slope of the signal corresponds to the change in H_2O_2 concentration and hence the activity of NADPH oxidase. Right after fMLP addition we observed oscillations (Fig. 2C), with a similar period and similar damping to those obtained with luminol. The exact period of the oscillations was difficult to determine with the same accuracy as with the luminol assay because estimates of the derivative will always be noisier than the original signal.

Table 1
Differential equations of the model.

Equation
$\frac{ds}{dt} = -k0 \cdot s$
$\frac{da}{dt} = k1 \cdot s - \frac{k2 \cdot a \cdot c \cdot s}{Km2 + a}$
$\frac{db}{dt} = f \cdot \frac{k2 \cdot a \cdot c \cdot s}{Km2 + a} - k3 \cdot b$
$\frac{dc}{dt} = \frac{k4 \cdot s \cdot b}{Km4 + b} - \frac{k5 \cdot c}{Km5 + c}$

Table 2
Model parameters.

Constant	Value
$k0$	0.005776 1/s
$k1$	0.2 1/s
$k2$	10 1/s
$Km2$	5
f	1
$k3$	2 1/s
$k4$	1.5×10^3 1/s
$Km4$	50
$Km5$	2
$k5$	20 1/s

Total H_2O_2 production is reduced when the glucose concentration is below 5 mM, but the total production of H_2O_2 production is not increased further when the glucose concentration exceeds 5 mM [29]. We have tested the impact of different glucose concentrations on the oscillations (Fig. 1C and D). It appears that using a medium without glucose (Fig. 1C) or increasing the glucose concentration to 10 mM (Fig. 1D) has no effect on the period or the number of oscillations compared to oscillations at a physiological glucose level around 5 mM (Fig. 1B). However, the amount of H_2O_2 decreases at lower glucose concentrations, which is in accordance with previous observations [29]. To support that glycolysis is not involved in the oscillations we added 2-deoxyglucose (2-DOG), which is phosphorylated by hexokinase. Phosphorylated 2-DOG cannot be metabolized further, and the cell is therefore depleted of ATP [30,31]. When we incubated cells with 2-DOG for 10 min before stimulation we did not observe any change in the oscillations in H_2O_2 production, which indicates that glycolysis is not involved in the oscillations.

3.2. NAD(P)H oscillations and Ca^{2+} response

NADPH oxidase oxidizes cytoplasmic NADPH and reduces oxygen on the other side of the membrane to superoxide, which spontaneously dismutates into H_2O_2 and O_2 [3,4,32]. We therefore expected that the oscillations in H_2O_2 production would be accompanied by oscillations in intracellular NADPH. We detected NADPH using its auto-fluorescence by excitation at 366 nm and emission at 450 nm in suspensions with a density of 5×10^6 cells/ml (Fig. 3). An increased cell density was used in order to increase the signal to noise ratio. Since NADH will also contribute to the auto-fluorescence we shall refer to the combined fluorescence due to NADPH and NADH as NAD(P)H fluorescence. When we stimulated the cells with PMA after pretreatment with wortmannin as in Fig. 1 we observed a slight increase in NAD(P)H fluorescence followed by a decrease that occurred around the same time as the H_2O_2 production peaks (Fig. 3A). We cannot determine if this decrease in NAD(P)H fluorescence is due to NADH or to NADPH. Upon stimulation with 100 nM fMLP the fluorescence decreased instantaneously (Fig. 3A) corresponding to an immediate decrease in the level of reduced NAD(P). Following that, oscillations were observed with a period identical to the period of oscillations in H_2O_2 (Fig. 3B). The oscillations in NAD(P)H (Fig. 3B) were about 180° out of phase with the H_2O_2 oscillations (Fig. 1B), which is evident from the phase plot of H_2O_2 luminol luminescence versus NADPH fluorescence (Fig. 3C). This phase difference is expected if the oscillations in fluorescence are due to oscillations in NADPH oxidase, because an increase in H_2O_2 production should be accompanied by a higher consumption of NADPH and hence a decrease in NAD(P)H fluorescence.

Oscillations in Ca^{2+} are seen in many different cells [33], but they have not been observed in a suspension of neutrophils before [5,7]. We measured the changes in intracellular Ca^{2+} following stimulation that induces oscillations in H_2O_2 production and NAD(P)H. Using Fura2 we did not observe oscillations in Ca^{2+}, in accordance with

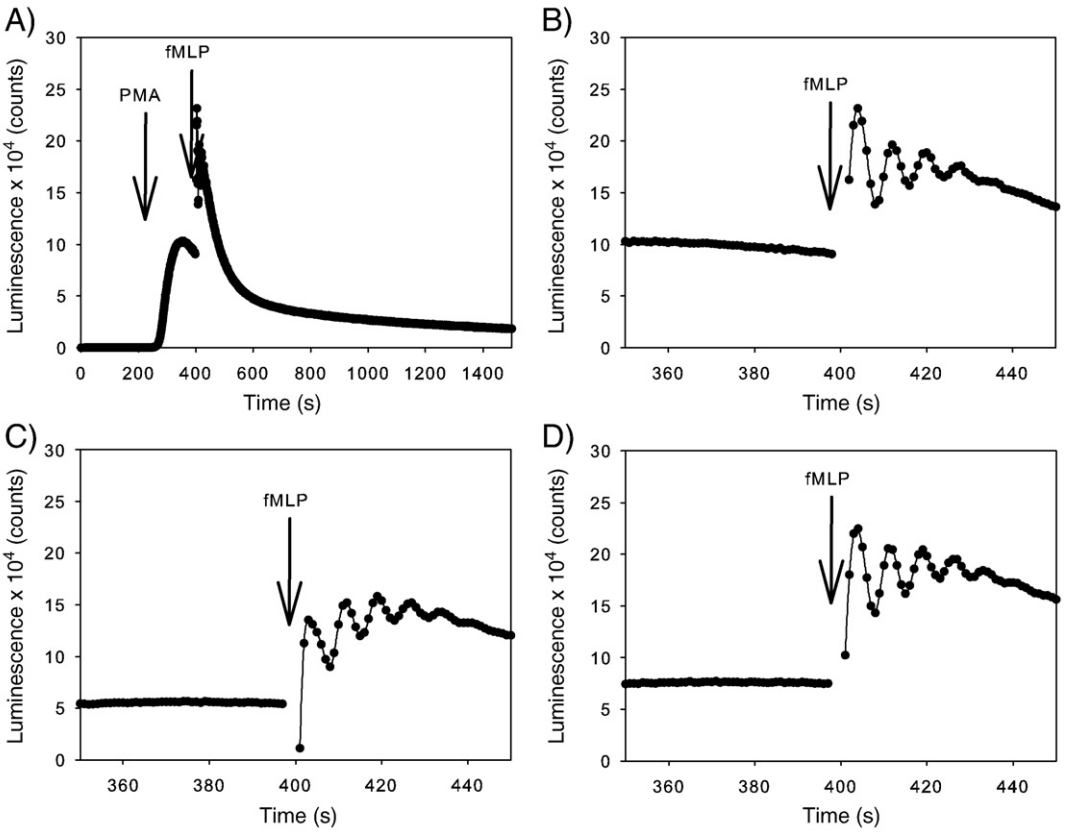

Fig. 1. Oscillations in H_2O_2 production. Neutrophils (10^6 cells/ml) were suspended in (A,B) buffer with 5 mM glucose. Panel B highlights the oscillatory part of panel A. The cells were preincubated at 37 °C for 10 min with 1 μM wortmannin, 10 μM luminol, 0.1 μM NaN_3 and 9 μg/ml HRP and subsequently stimulated with 3.25 nM PMA and 3 min after that with 100 nM fMLP (marked with arrows). In addition we also induced oscillations in neutrophils that were suspended in a buffer without glucose (C), or with 10 mM glucose (D).

previous measurements of intracellular Ca^{2+} [5,7], but not consistent with measurements of Ca^{2+} in single neutrophils following adherence and stimulation with fMLP [10]. We also measured intracellular Ca^{2+} with Fluo4 and Fluo5-F, which both have a higher K_d for Ca^{2+} than Fura2, and again we did not observe any oscillations in intracellular Ca^{2+}.

The plasma membrane potential of neutrophils is affected when they are stimulated due to the electrogenic properties of NADPH oxidase [24,34], and we speculated if the oscillations in NADPH oxidase could induce oscillations in the membrane potential. We measured the membrane potentials as described in Experimental procedures, but we did not observe oscillations in the fluorescence of any of the three probes used to measure the membrane potential. This is in contradiction with experiments on single neutrophils by Petty and co-workers that have reported periodic sustained oscillations in the membrane potential following adherence to a surface [8].

3.3. Synchronization of the oscillations between individual cells, effect of cell density

When measuring macroscopic damped oscillations in a suspension of cells the question always arises of whether the individual cells in the suspension oscillate in phase due to a compound that locks their phase or if the damping of the oscillations is caused by individual cells exhibiting sustained oscillations but which slowly drift out of phase. A good example is oscillating glycolysis in the yeast *Saccharomyces cerevisiae* [13,14,35]. Here acetaldehyde acts as a synchronizing molecule diffusing rapidly between the cells and thereby maintains perfect synchrony between individual oscillating cells. If the density of yeast cells becomes lower than a critical density the oscillations disappear because acetaldehyde becomes too diluted [13,36].

In order to investigate whether the same is true for neutrophils that oscillate in suspension we induced oscillations at different cell densities, ranging from 5×10^6 to 5×10^4 cells/ml (Fig. 4). We analyzed the dilution experiments by calculating the decay exponent of the oscillations. The decay exponent was found by averaging the decaying damped oscillation and then fitting an exponential function through the maxima (Fig. 4). We found that the magnitude of the exponent of decay was reproducible between different donors and that it was independent of the density (5×10^4 to 5×10^6 cells/ml) of the neutrophils. According to the theory of coupled oscillators [13,36,37] an exponent of decay, which is independent of changes in cell density of several orders of magnitude, signifies the absence of synchronization between individual cells. Hence, since the exponent of decay is insensitive to changes in density of two orders of magnitude, we may conclude that the cells in suspension are not synchronized.

3.4. Inhibition with 1-butanol

The exact mechanism causing the oscillations in H_2O_2 production remains unknown, but it has been shown that the α subunit of a G-protein coupled receptor is crucial for induction of oscillations in H_2O_2 production and in shape/size, and that general PKC activity is only necessary for oscillations in H_2O_2 production [5]. As we did not observe oscillations in Ca^{2+} we speculated that oscillations in diacylglycerol (DAG) could be involved in mediating the oscillations in H_2O_2 production. PKC δ is a PKC isoform that is independent of Ca^{2+} and regulated by DAG. It is crucial for the activation of NADPH oxidase following fMLP stimulation whereas the corresponding activation of NADPH oxidase by PMA is almost independent of PKC δ [38,39]. Rottlerin is a non-specific inhibitor of PKC δ [38,40], and we measured

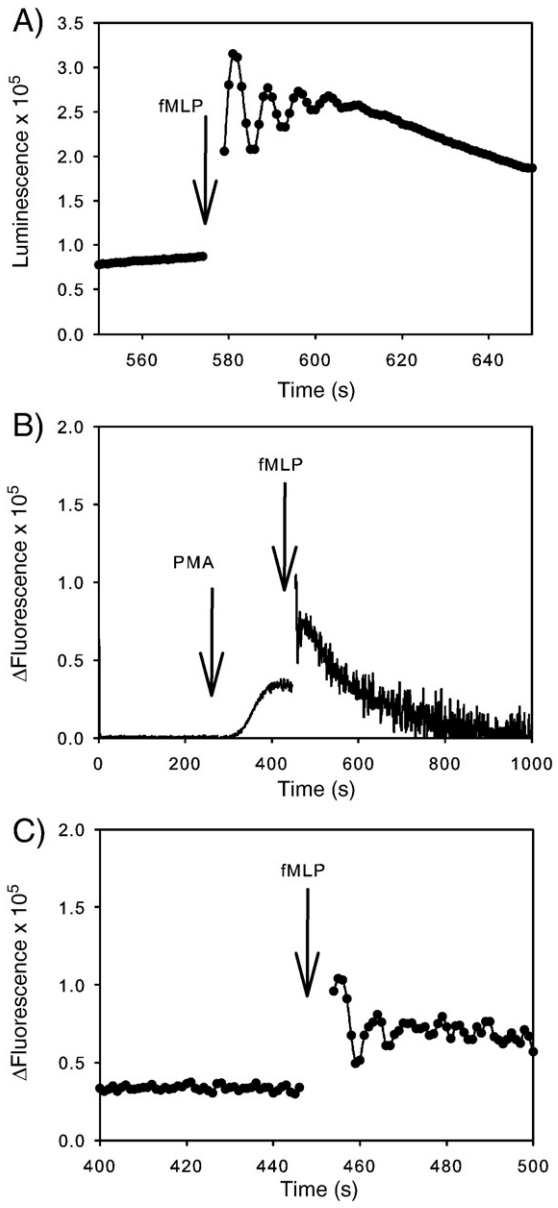

Fig. 2. Alternative conditions for oscillations. (A) The cells were preincubated at 37 °C for 10 min with 10 μM luminol, 0.1 μM NaN_3 and 9 μg/ml HRP and subsequently stimulated with 1.6 nM PMA and 4 min after that with 10 nM fMLP (marked with arrow). Oscillations in H_2O_2 production detected with Amplex Red (B,C). Neutrophils (10^6 cells/ml) were suspended in HEPES buffer with 5 mM glucose. The cells were preincubated at 37 °C for 10 min with 50 μM Amplex Red, 1 mM NaN_3, 5.24 μg/ml HRP together with 1 μM wortmannin and subsequently stimulated with 3.25 nM PMA and 3 min after that with 100 nM fMLP (marked with arrows). (B and C) From the Amplex red signal we found the change in H_2O_2 production by differentiating the signal from Amplex Red using the three point method (see text).

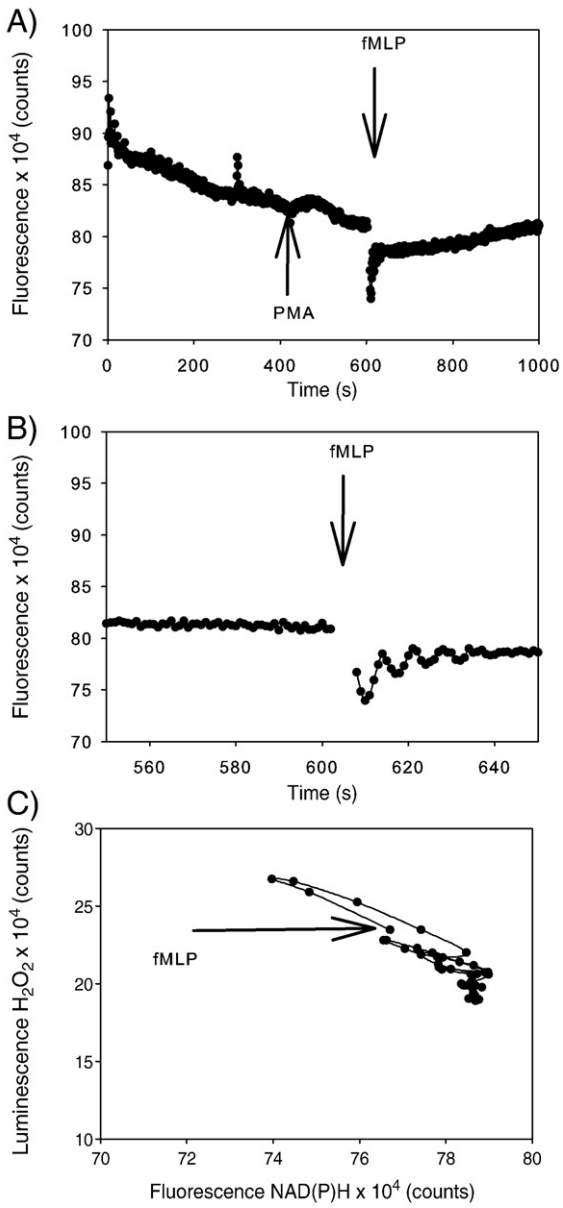

Fig. 3. NAD(P)H oscillations. NAD(P)H changes upon induction of oscillations were measured as the auto-fluorescence at 450 nm with excitation at 366 nm. (A) The neutrophils (5×10^6 cells/ml) were suspended in a buffer with 5 mM glucose and preincubated at 37 °C for 10 min with 1 μM wortmannin and subsequently stimulated with 3.25 nM PMA and 3 min after that with 100 nM fMLP (marked with arrows). Panel B is from the part of the experiment where we added fMLP. (C) The luminescence from H_2O_2 obtained with cells from the same donor is plotted as a function of the intensity from NAD(P)H fluorescence following wortmannin incubation and additional PMA and fMLP stimulation. This phase diagram shows that the oscillations in H_2O_2 are about 180° out of phase with the NAD(P)H oscillations.

the H_2O_2 production in neutrophils incubated with 10 μM of rottlerin. When we incubated the cells with 10 μM rottlerin, in addition to wortmannin, H_2O_2 production following PMA addition was almost unchanged, but when we added fMLP 3 min later we did not observe any additional response and hence also no oscillations in H_2O_2 formation (results not shown). This finding is in agreement with previous suggestions that PKC δ is involved in activation of NADPH oxidase after fMLP stimulation [38], and it suggests that the oscillations in NADPH oxidase activity involve PKC δ. However, it has also been shown that rottlerin at this concentration is not specific and that it may also inhibit other cell signaling pathways [40].

In order to obtain further information about the potential involvement of DAG in the regulation of H_2O_2 production we treated the neutrophils with 1-butanol, which inhibits PLD, and its non-inhibitory analog 2-butanol as control [41,42]. Moderate inhibition [41,42] with 0.3% 1-butanol decreased the amplitude and the number of oscillations (Fig. 5A), whereas the control showed only little effect (Fig. 5B). We also calculated the damping factor, defined as the ratio between the first and second amplitude of the oscillations (Fig. 5C). Here we define the first amplitude as the difference between the first maximum and minimum, and the second amplitude as the difference between the second maximum and minimum. This factor is reduced when the cells have been incubated with 1-butanol suggesting that the oscillations in NADPH oxidase activity involve PLD. An oscillating

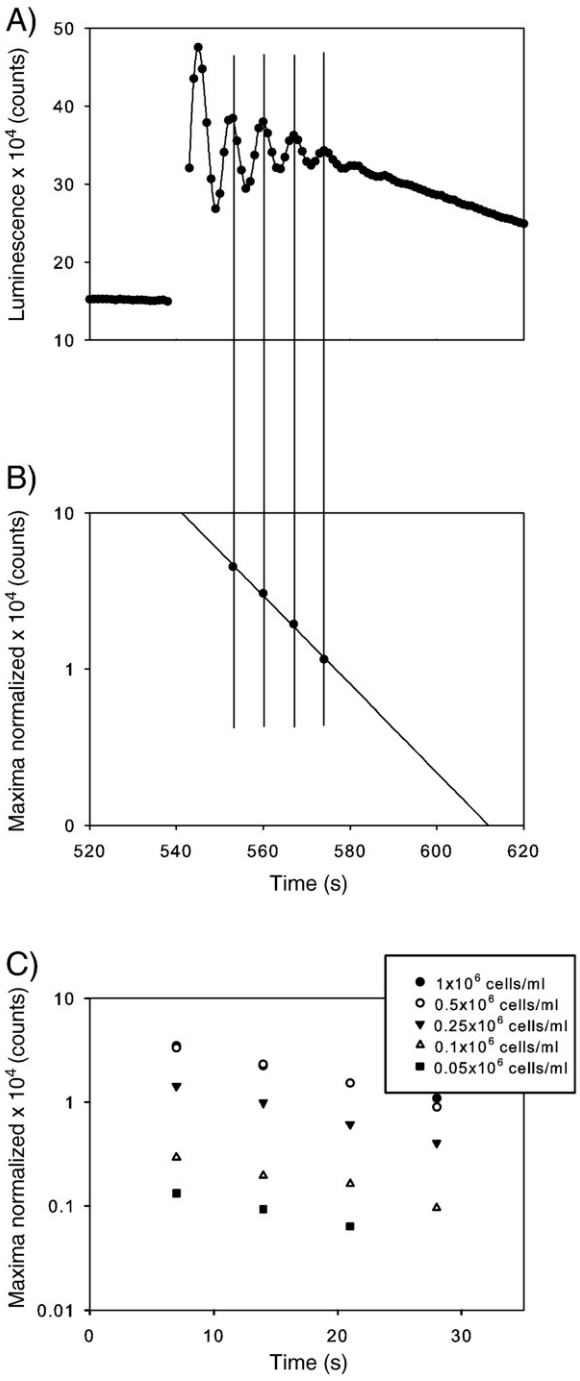

Fig. 4. The effect of cell density on the oscillations. The effect of neutrophil density on the oscillations was tested by inducing oscillations in suspensions of neutrophils ranging from 5×10^4 to 1×10^6 cells/ml. The oscillations were induced as in Fig. 1. An experiment with a cell density of 10^6 cells/ml where the neutrophils are stimulated with fMLP. (A) The maxima in the oscillatory trace are normalized according to the mean value of the period and plotted in (B). An exponential function is then fitted through the points representing the normalized maxima and the decay exponent is given as the exponential coefficient. The normalized maxima for different densities are shown in (C).

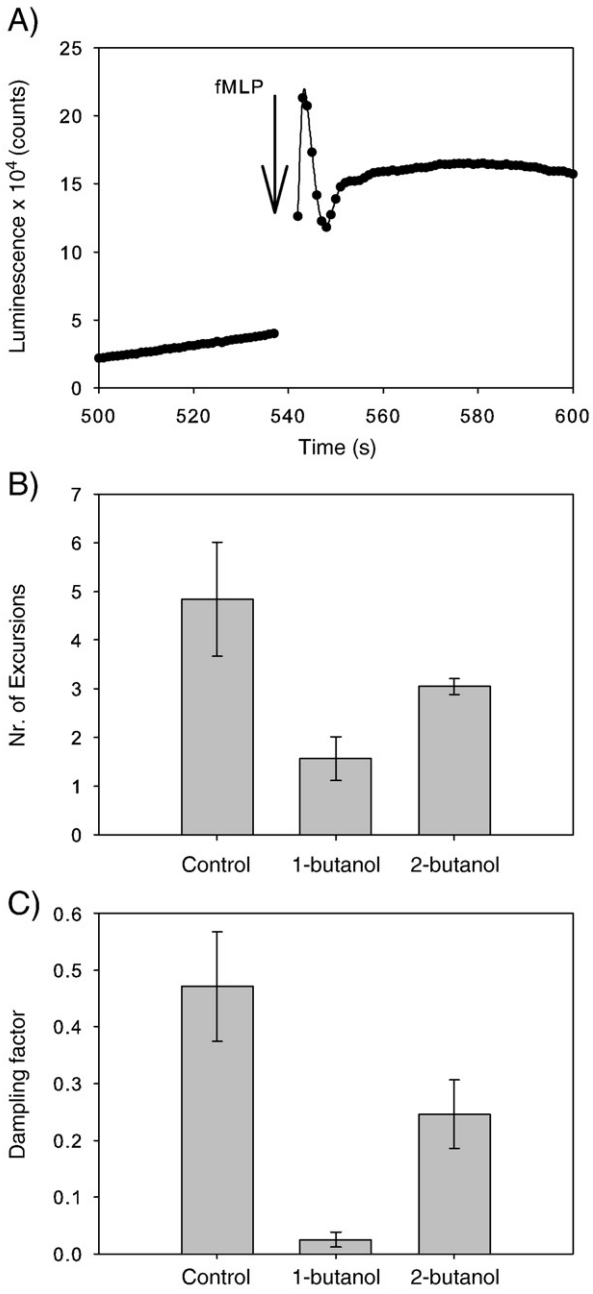

Fig. 5. Inhibition of phospholipase D. The neutrophils were incubated with 0.3% 1-butanol 10 min prior to PMA stimulation at same conditions as in Fig. 1 (A). The number of excursions is shown when the neutrophils are incubated with 0.3% 1-butanol, 0.3% of the non-inhibitory analog 2-butanol and without alcohols (B). The damping factor which corresponds to the ratio between the first and second amplitude is computed for the three different conditions (C). The neutrophils were preincubated at 37 °C for 10 min with 1 μM wortmannin, 10 μM luminol, 0.1 μM NaN_3 and 9 μg/ml HRP and subsequently stimulated with 3.25 nM PMA and 3 min after that with 100 nM fMLP (marked with arrow).

PLD activity would imply that the concentration of DAG may also oscillate.

3.5. Mathematical model of the oscillations in H_2O_2 production

Many biological systems are known to oscillate [33] and the neutrophils appear to be no exception. In order to support the experimental observations of oscillations in H_2O_2 production in neutrophils we have formulated and simulated a simple mathematical model based on the following experimental findings (see Fig. 6 for an illustration): The dynamic changes in shape and size are caused by oscillations in F-actin [23,43]. The dynamics of F-actin, in turn, are regulated by Rac and PIP_3 (the product of PI3K) among other molecules [44–46]. Furthermore, the activity of PLD is activated by PIP_3, and some isoforms of PLD are also activated by

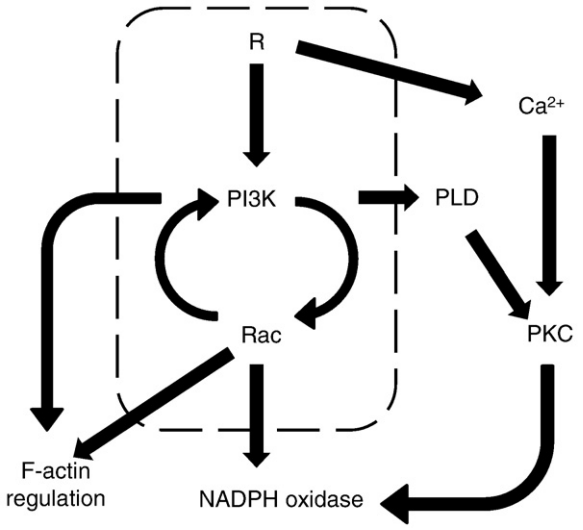

Fig. 6. Schematic model of the system. The figure illustrates the interactions between the different components. The scheme is explained in details in text and is based on data from ref. [5,44–53]. R (receptor), PI3K (phosphoinositide 3-kinases), PLD (phospholipase D), PKC (protein kinase C).

Rac [47]. The activity of PI3K is important for the oscillations. PI3K is activated by fMLP [48] and oscillations can only be observed following addition of low doses of fMLP or high doses of fMLP when the PI3K inhibitor wortmannin or 17-hydroxywortmannin has been added at specific doses [5]. Despite the fact that wortmannin is a potent inhibitor of PI3K, residual activity (1–5%) is still observed following addition of 0.1–1 μM wortmannin [49,50]. Rac was found to be activated downstream of PI3K, but at the same time PI3K has also been found to be activated by Rac [44]. This positive feedback loop is likely to be important in neutrophil activation as it will amplify the signal [44,45,51]. Other isoforms of PKC are in addition also activated by Ca^{2+}, which is also triggered following fMLP stimulation [52,53].

We have constructed a mathematical model with feedback loops between PI3K and Rac, which is emphasized with a box in Fig. 6. With this model we aim at describing the interactions we think are central for the elements in the oscillations and we have not included NADPH oxidase activity and the amount of F-actin because they are not the direct cause of the oscillations. However, both F-actin polymerization and NADPH oxidase are regulated directly from this feedback loop as shown in Fig. 6. The model is dimensionless with respect to concentrations and consists of four variables s, a, b and c. Variable a corresponds to inactive PI3K, while variable b corresponds to active PI3K. Variable c then corresponds to Rac. Variable s represents the transient signal that is induced by binding of fMLP to the fMLP receptor. The model's differential equations are listed in Table 1 while the parameters used in the simulations are listed in Table 2.

$s\rightarrow$ (*Decay of signal from receptor*)

Variable s decays exponentially with a rate that is proportional to s. Variable a is generated with a rate proportional to s and is converted into b.

$\rightarrow a$ (*Formation of inactive PI3K*)

$a\rightarrow b$ (*PI3K activation*)

The transition from a to b is described using Henri–Michaelis–Menten kinetics where c and s act as activators; b is broken down in a simple first order reaction with the rate constant $k3$.

$b\rightarrow$ (*PI3K decay*)

The factor f in the rate term describes the relative change in concentration due to translocation of the active PI3K to the membrane [54]. The variable c is generated with a rate expression described by Henri–Michaelis–Menten kinetics dependent on b and s.

$\rightarrow c$ (*Rac activation*)

Finally, c is degraded in a Henri–Michaelis–Menten type reaction.

$c\rightarrow$ (*Rac inactivation*)

The mechanistic background of Eqs. (1) and (2) in Table 1 is that PI3K is continually activated and deactivated, and we assume here that the activation is mediated by Rac. The justification for Eq. (3) in Table 1 is that active PI3K is activating Rac [44,45].

We have explored the stability of the model using different approaches. The initial conditions can be changed with a factor of 10 without affecting the result, with exception of s. If the parameters are changed by ±10% the qualitative behavior (i.e. damped oscillations) remains the same and the period changes at most 5–15%. If s is constant ($k0=0$), damped oscillations are still observed and if $k1$ is 0.4 s^{-1} and $k2$ is 4 s^{-1} the model shows sustained oscillations. The variable s decreases slowly compared to the damping of the oscillations (Fig. 7D). Because s is not constant we have computed the three eigenvalues for $s=1$, and we found that one eigenvalue is real and negative and that the other two form a complex pair with negative real part, thus the fixed point is a stable focus. To test if the oscillations are a property of the biological network or just due to Michaelis–Menten terms in the model, we have converted all the Michaelis–Menten terms (e.g. $(k2\cdot a\cdot c\cdot s/(Km2+a))$) into terms that only are based on the product of the variables (e.g. $(k2^*\cdot a\cdot c\cdot s)$). The reduced model still showed damped oscillations with the same period (not shown), which is another indication that the positive feedback loop in Fig. 6 is very likely to oscillate. Computation of the elasticities of individual rates of the model [55] gave values of 0 or 0.998 to 1 (data not shown). This confirms that it is the model structure and not the form of the individual rate expressions that determines the dynamics.

Though the model is qualitative, because the details of the interactions of PI3K and Rac still have to be elucidated, it is able to explain most of the oscillatory behavior exhibited by neutrophils in suspension. The model predicts that damped oscillations can occur for the set of parameters given in Table 2 (Fig. 7A). The period of the oscillation is 8 s, in agreement with the period determined in the experiments. Wortmannin inhibits the activity of PI3K, which in this model is reflected by $k4$. If $k4$ is increased (less inhibition by wortmannin) the oscillations essentially disappear (Fig. 7B). If $k4$ is decreased by a factor of 10 the oscillations disappear completely and b becomes close to zero (data not shown). This reflects the experimental observations that the oscillations are dependent on PI3K activity. The model can also simulate oscillations when PI3K is not inhibited by wortmannin. If $k4$ is increased by a factor of 50 as in Fig. 7B, the model still shows oscillations if $k1$ (the signal from the receptor), is decreased by a factor of 50 (data not shown). That simulation corresponds to the experiment in Fig. 2A where wortmannin is omitted and the amount of fMLP is reduced.

Cells are not just bags of enzymes, in fact most processes are organized spatially in the cell. PI3K is orientated in the plasma membrane according to the polarity of the neutrophil and the localization of PI3K has been suggested to be associated with an enhanced feedback from Rac since this should increase the local concentration of both PI3K and Rac [44]. Conversely, inhibition of polarization will lead to a decrease in the local concentrations of PI3K, which we model by decreasing f. As illustrated in Fig. 7C a decrease in f to 0.6 leads to an increase in the period of the oscillations. This can be

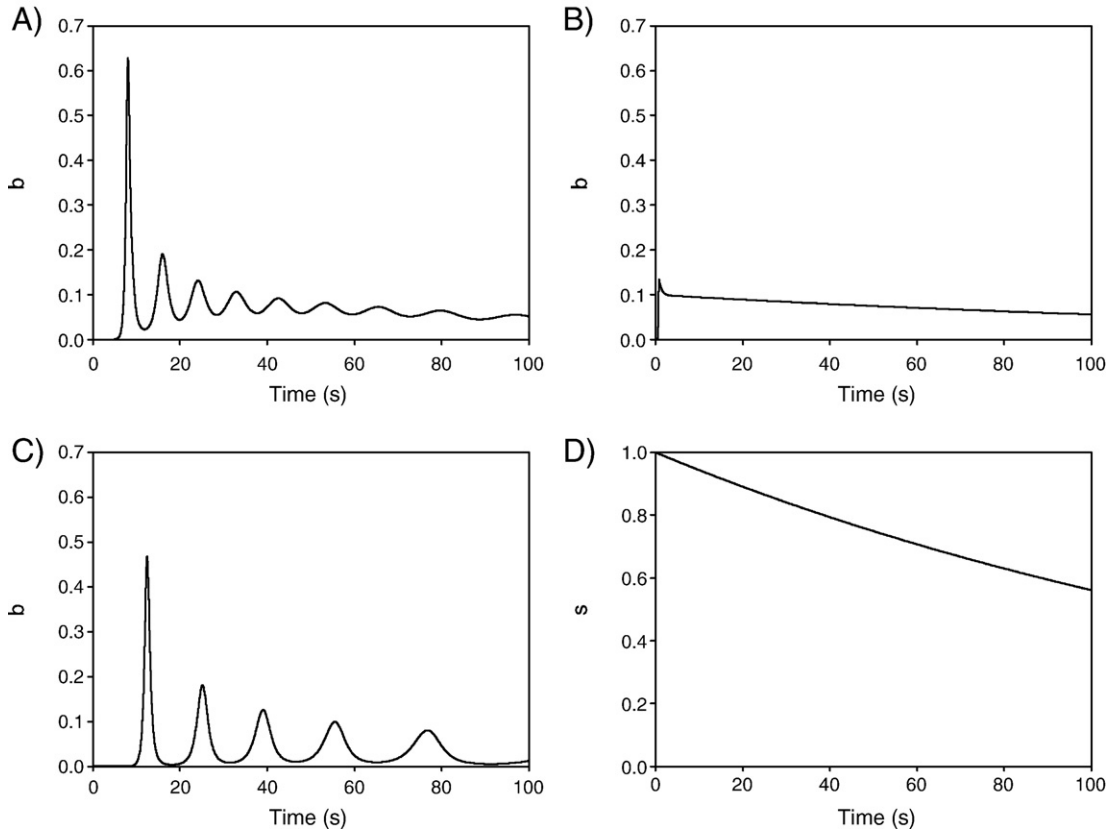

Fig. 7. Simulations of oscillations. (A) Simulation of the model described in Tables 1 and 2. The initial values for the variables *a*, *b* and *c* were 0.01, 0.0001 and 0.0001 respectively. We simulated the lack of wortmannin inhibition by increasing *k*4 to 7.5×10^4 1/s (B), which blocks the oscillations. (C) If *f* is decreased to 0.6 the period of the oscillations increases. (D) The decay of *s*.

tested experimentally because addition of cytochalasin B blocks actin filament assembling and neutrophils become unable to generate polarized pseudopodia [56]. Therefore, we would expect that addition of cytochalasin B to the cells will increase the period of the oscillations in the H_2O_2 production.

The present simulations demonstrate that the oscillations in H_2O_2 production and F-actin may be controlled by a core oscillator that controls both processes concurrently. The model can be extended with a few extra reactions to include the reactions responsible for actin polymerization and H_2O_2 production in order to simulate the oscillations in shape and size together with NADPH oxidase activity. The resulting model (work in progress) will have the ability to simulate the oscillations in H_2O_2 production and oscillations in shape and size as two oscillators coupled by a central element, such that in the intact system the two processes oscillate with the same frequency. Inhibiting either of these processes may lead to the knockout of both oscillators or only one of them at the time, depending on the site of inhibition.

3.6. Inhibition with cytochalasin B

The mathematical model predicts that cytochalasin B potentially could influence the period of the oscillations. Cytochalasin B inhibits actin polymerization and 10 µM cytochalasin B blocks changes (including oscillations) in shape and size [23]. However, when we incubated the cells with 5 or 10 µM cytochalasin B, in addition to wortmannin, we still observed oscillations in H_2O_2 production, but the period of the oscillations increased to 11.3 ± 0.5 s (Fig. 8C). Conversely, as expected we did not observe any oscillations in shape or size (Fig. 8D) compared to when the neutrophils were not inhibited with cytochalasin B (Fig. 8B). This indicates that the oscillations in shape and size can be decoupled from the oscillations in H_2O_2 formation, but decoupling is associated with a significant change in oscillation period of H_2O_2 generation. These experimental observations are in line with the predictions of the model. We obtained identical results with 10 µM cytochalasin B.

4. Discussion

We have investigated oscillations in H_2O_2 generation and fluorescence that can be ascribed to NAD(P)H in neutrophils in suspension. We have shown that the oscillations in NAD(P)H fluorescence accompany the oscillations in H_2O_2 production in neutrophils upon treatment of the cells with wortmannin followed by stimulation with PMA and fMLP. Together with the Amplex Red results this clearly demonstrates that the oscillations in H_2O_2 production are associated with NADPH oxidase activity and not with other factors in the H_2O_2 assay. However, we have not been able to find oscillations in intracellular Ca^{2+} and plasma membrane potential accompanying the oscillations in NAD(P)H and H_2O_2 production as it was claimed for single cells [8–10]. Furthermore, we have investigated the effect of extracellular glucose concentration on the oscillations and we have found that neither the frequency nor the amplitude is markedly affected. This is partly in contradiction to results obtained with single isolated neutrophils, which suggest that unstimulated adhered neutrophils are able to oscillate and that high glucose levels are able to increase the frequency of the oscillations in NAD(P)H, H_2O_2 and Ca^{2+} [9]. In the single cell studies, sustained Ca^{2+} oscillations that coexist with sustained NAD(P)H oscillations were also reported [10]. However, consistent with results reported by others [5,7], we have not been able to detect any Ca^{2+} oscillations in the neutrophils in suspension. It is worth noting here that some of the previous results showing oscillations and waves of Ca^{2+} in single

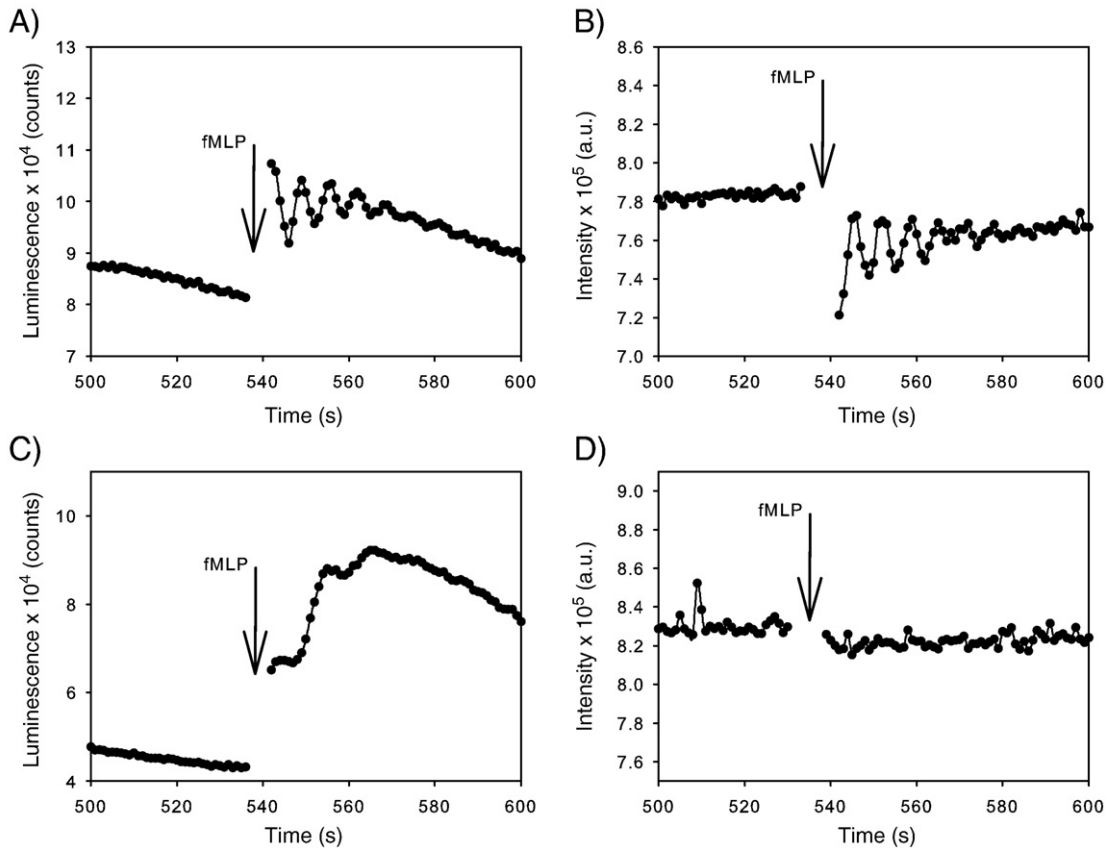

Fig. 8. Inhibition with cytochalasin B. H_2O_2 was measured as in Fig. 1 and light scattering was measured with 636.2 nm pulsed picosecond diode laser, where the neutrophils were preincubated with 1 μM wortmannin 10 min prior 3.25 nM PMA stimulation. (A) H_2O_2 production in control cells without cytochalasin B and (B) oscillations in light scattering from cells without cytochalasin B. (C) H_2O_2 production from cells incubated with 5 μM cytochalasin B in addition to 1 μM wortmannin 10 min prior to PMA stimulation and (D) light scattering from cells that also were incubated with 5 μM cytochalasin B.

neutrophils were recently retracted [57] and others have raised doubt about their existence [11,12].

Oscillations in NAD(P)H fluorescence similar to those observed here have been observed in suspensions of yeast cells. In yeast cells the period of oscillation is 35–40 s and the oscillations represent a collective phenomenon. The latter can be inferred from the observation that a single isolated yeast cell is unable to oscillate upon addition of glucose [14], whereas a suspension of yeast cells shows essentially undamped oscillations where all cells oscillate in phase when the cell density surpasses a certain threshold [13]. In the suspension, acetaldehyde is serving as a synchronizing agent between individual cells, and hence the density of the suspension becomes critical in maintaining synchronized oscillations [13,14]. If the cell density is too low the amount of acetaldehyde secreted into the external volume becomes too low to synchronize the cells' metabolism. Here, we found that the oscillations in neutrophils are independent of the cell density over a wide range of cell densities (change in cell density by a factor 10^2) (Fig. 4). When this requirement is fulfilled theoretical work on coupled oscillators [36,37] assures that the oscillations are not communicated by an extracellular compound. This leaves us with two alternative explanations for why the oscillations in H_2O_2 production and NAD(P)H are damped. (i) It could be that the individual neutrophils in the suspension show sustained oscillations, but that they drift out of phase because they lack a synchronizing agent, or (ii) it is possible that all the cells in suspension exhibit a damped oscillation. The mathematical model shows damped oscillations, which are caused by a combination of a stable fixed point and a decreasing signal from the receptor (s). The model therefore supports the later hypothesis namely that each cell shows damped oscillations and that the cells are uncoupled.

There has been much speculation about the source of the oscillations in neutrophils. In single neutrophils the mechanism of oscillation has been suggested to be caused by oscillations in Ca^{2+}, glycolysis [9,10] or an oscillating reaction driven by myeloperoxidase [58,59]. The oscillations that are observed in suspension were shown to be related to oscillations in shape and size and it has been suggested that they are associated with the cyclic changes in shape that neutrophils undergo during migration [5,23]. When the neutrophils are not incubated with wortmannin the oscillations are induced by about 10 nM fMLP which is also the dose of fMLP that induces the optimal migration speed [60–62]. Violin et al. [63] have shown that protein kinase C (PKC) can operate in an oscillatory mode with a period of around 1 min at room temperature. At 30 °C the period of H_2O_2 production in neutrophils was reported to be about 15 s [5], and we have found that at 25 °C the period of the oscillations is about 30 s (data not shown). This is roughly the same period as obtained with shape oscillations at 25 °C [23]. There is evidence suggesting that the oscillations in H_2O_2 production and shape oscillations depend on the G-protein coupled receptor and that PKC is only crucial for the oscillations in H_2O_2 production [5]. When we added 10 μM rottlerin to a suspension of neutrophils we observed a response when we stimulated the cells with PMA, but when we later added fMLP we observed essentially no response and a complete loss of the oscillations in H_2O_2 production. Rottlerin is supposed to inhibit a Ca^{2+}-insensitive isoform of PKC (PKC δ), but it will also inhibit a range of other proteins [40]. As PKC δ is insensitive to Ca^{2+} and only sensitive to DAG this indicates that the oscillations might be caused by enzyme-catalyzed reactions upstream of DAG (e.g. PLD) and hence that oscillations in H_2O_2 formation should be accompanied by oscillations in DAG. When we inhibited PLD with 1-butanol (Fig. 5)

the oscillations were inhibited in a similar fashion, which supports the fact that the oscillations in H_2O_2 production could be due to oscillations in DAG. The oscillations in H_2O_2 production were not annihilated by cytochalasin B, although cytochalasin B at concentrations we have used is known to inhibit oscillations in morphology [23]. However, the period of the oscillations in H_2O_2 production was increased. This result clearly demonstrates that the oscillations in NADPH oxidase activity and those in morphology may be decoupled, suggesting that both are derived from a common master oscillator. We verified that the concentration of cytochalasin B used here completely blocks oscillations in shape and size (Fig. 8). We have also measured light scattering following incubation with 0.3% 1-butanol or 2-butanol, and to our surprise we saw that both 1-butanol and 2-butanol inhibited changes in cell shape and size. This is in accordance with previous observations showing that 1-butanol inhibits migration [64,65].

It has previously been suggested that the oscillations in NADPH oxidase activity are driven and controlled by oscillations in F-actin by a direct physical contact [5]. However, our results with cytochalasin B and butanol do not support that hypothesis, because we can decouple these two events. Conversely it was demonstrated that oscillations in NADPH oxidase could be inhibited with the inhibitor of Ca^{2+}-dependent protein kinases staurosporine without affecting the oscillations in shape and size [5]. Many signal transduction pathways are regulated through positive and negative feedback control, which in turn induce oscillations in the cascade [66,67]. To facilitate our understanding of the oscillations and their mechanistic background we have constructed a mathematical model of the interplay between PI3K and Rac. Though the model is qualitative and was intended to test our hypothesis and generate new perspectives, it is stable and robust towards perturbations as judged from the sensitivity analysis. The interplay that is modeled with the mathematical model could form the core of a master oscillator in the cell that drives both the oscillations in NADPH oxidase activity and the oscillations in shape and size of the cells. This assumption is justified by the fact that oscillations in NADPH oxidase activity may be decoupled from the oscillations in shape and size. The model involves the activity of PI3K, which is consistent with the known effect of wortmannin. The effect of cytochalasin B on the period of the oscillations can be explained by a lack of cell polarity and organization, which reduces the activity of the processes leading to activation of PI3K and Rac.

Acknowledgments

This research was funded by the Danish Natural Science Research Council (grant nos 272-06-0345 and 272-08-150). The authors would like to thank Mrs. Anita Lunding for technical assistance and the staff at the Blood Bank (Odense University Hospital) for withdrawing the blood from donors. We thank Drs. Maurice B. Hallett and Ursula Kummer for comments and discussion.

References

[1] C. Nathan, Neutrophils and immunity: challenges and opportunities, Nat. Rev. Immunol. 6 (2006) 173–182.
[2] S.J. Weiss, Tissue destruction by neutrophils, N. Engl. J. Med. 320 (1989) 365–376.
[3] R.K. Root, J.A. Metcalf, H_2O_2 release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H_2O_2: studies with normal and cytochalasin B-treated cells, J. Clin. Investig. 60 (1977) 1266–1279.
[4] R.K. Root, M.S. Cohen, The microbicidal mechanisms of human neutrophils and eosinophils, Rev. Infect. Dis. 3 (1981) 565–598.
[5] M.P. Wymann, P. Kernen, D.A. Deranleau, M. Baggiolini, Respiratory burst oscillations in human neutrophils and their correlation with fluctuations in apparent cell shape, J. Biol. Chem. 264 (1989) 15829–15834.
[6] M.P. Wymann, P. Kernen, D.A. Deranleau, B. Dewald, V. von Tscharner, M. Baggiolini, Oscillatory motion in human neutrophils responding to chemotactic stimuli, Biochem. Biophys. Res. Commun. 147 (1987) 361–368.
[7] G.M. Omann, M.M. Porasik, L.A. Sklar, Oscillating actin polymerization/depolymerization responses in human polymorphonuclear leukocytes, J. Biol. Chem. 264 (1989) 16355–16358.
[8] A.L. Kindzelskii, H.R. Petty, Ion channel clustering enhances weak electric field detection by neutrophils: apparent roles of SKF96365-sensitive cation channels and myeloperoxidase trafficking in cellular responses, Eur. Biophys. J. 35 (2005) 1–26.
[9] U. Kummer, J. Zobeley, J.C. Brasen, R. Fahmy, A.L. Kindzelskii, A.R. Petty, A.J. Clark, H.R. Petty, Elevated glucose concentrations promote receptor-independent activation of adherent human neutrophils: an experimental and computational approach, Biophys. J. 92 (2007) 2597–2607.
[10] H.R. Petty, Neutrophil oscillations: temporal and spatiotemporal aspects of cell behavior, Immunol. Res. 23 (2001) 85–94.
[11] O. Slaby, D. Lebiedz, Oscillatory NAD(P)H waves and calcium oscillations in neutrophils? A modeling study of feasibility, Biophys. J. 96 (2009) 417–428.
[12] M.B. Hallett, D. Lebiedz, M.S. Mommer, C. Reble, E.J. Saltmarsh, Fantastic Ca^{2+} "z-waves" fade out quietly, Cell Calcium 46 (2009) 85–86.
[13] S. De Monte, F. Test, Dynamical quorum sensing: population density encoded in cellular dynamics, Proc. Natl Acad. Sci. U. S. A. 104 (2007) 18377–18381.
[14] A.K. Poulsen, M.O. Petersen, L.F. Olsen, Single cell studies and simulation of cell–cell interactions using oscillating glycolysis in yeast cells, Biophys. Chem. 125 (2007) 275–280.
[15] K. Theilgaard-Monch, J. Cowland, N. Borregaard, Profiling of gene expression in individual hematopoietic cells by global mRNA amplification and slot blot analysis, J. Immunol. Methods 252 (2001) 175–189.
[16] B.K. Rada, M. Geiszt, K. Kaldi, C. Timar, E. Ligeti, Dual role of phagocytic NADPH oxidase in bacterial killing, Blood 104 (2004) 2947–2953.
[17] B.E. Seligmann, E.K. Gallin, D.L. Martin, W. Shain, J.I. Gallin, Interaction of chemotactic factors with human polymorphonuclear leukocytes: studies using a membrane potential-sensitive cyanine dye, J. Membr. Biol. 52 (1980) 257–272.
[18] B.E. Seligmann, J.I. Gallin, Use of lipophilic probes of membrane potential to assess human neutrophil activation. Abnormality in chronic granulomatous disease, J. Clin. Investig. 66 (1980) 493–503.
[19] S.T. Smiley, M. Reers, C. Mottola-Hartshorn, M. Lin, A. Chen, T.W. Smith, G.D. Steele Jr., L.B. Chen, Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1, Proc. Natl Acad. Sci. U. S. A. 88 (1991) 3671–3675.
[20] M. Reers, T.W. Smith, L.B. Chen, J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential, Biochemistry 30 (1991) 4480–4486.
[21] F. Di Lisa, P.S. Blank, R. Colonna, G. Gambassi, H.S. Silverman, M.D. Stern, R.G. Hansford, Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition, J. Physiol. 486 (Pt 1) (1995) 1–13.
[22] S. Hoops, S. Sahle, R. Gauges, C. Lee, J. Pahle, N. Simus, M. Singhal, L. Xu, P. Mendes, U. Kummer, COPASI—a COmplex PAthway SImulator, Bioinformatics 22 (2006) 3067–3074.
[23] M.U. Ehrengruber, D.A. Deranleau, T.D. Coates, Shape oscillations of human neutrophil leukocytes: characterization and relationship to cell motility, J. Exp. Biol. 199 (1996) 741–747.
[24] T.E. Decoursey, E. Ligeti, Regulation and termination of NADPH oxidase activity, Cell. Mol. Life Sci. 62 (2005) 2173–2193.
[25] U. Kummer, M.J.B. Hauser, K. Wegmann, L.F. Olsen, G. Baier, Oscillations and complex dynamics in the peroxidase–oxidase reaction induced by naturally occurring aromatic substrates, J. Am. Chem. Soc. 119 (1997) 2084–2087.
[26] M.J.B. Hauser, L.F. Olsen, The role of naturally occurring phenols in inducing oscillations in the peroxidase–oxidase reaction, Biochemistry 37 (1998) 2458–2469.
[27] H. Degn, Compound 3 kinetics and chemiluminescence in oscillatory oxidation reactions catalyzed by horseradish peroxidase, Biochim. Biophys. Acta 180 (1969) 271–290.
[28] J. Faldt, M. Ridell, A. Karlsson, C. Dahlgren, The phagocyte chemiluminescence paradox: luminol can act as an inhibitor of neutrophil NADPH-oxidase activity, Luminescence 14 (1999) 153–160.
[29] A.S. Tan, N. Ahmed, M.V. Berridge, Acute regulation of glucose transport after activation of human peripheral blood neutrophils by phorbol myristate acetate, fMLP, and granulocyte-macrophage colony-stimulating factor, Blood 91 (1998) 649–655.
[30] A.K. Poulsen, A.Z. Andersen, J.C. Brasen, A.M. Scharff-Poulsen, L.F. Olsen, Probing glycolytic and membrane potential oscillations in *Saccharomyces cerevisiae*, Biochemistry 47 (2008) 7477–7484.
[31] M. Herve, J. Wietzerbin, S. Tran-Dinh, Non-cooperative effects of glucose and 2-deoxyglucose on their metabolism in *Saccharomyces cerevisiae* studied by 1H-NMR and 13C-NMR spectroscopy, Eur. J. Biochem. 218 (1993) 221–228.
[32] B.M. Babior, NADPH oxidase, Curr. Opin. Immunol. 16 (2004) 42–47.
[33] A. Goldbeter, Biochemical Oscillations and Cellular Rhythms, Cambridge University Press, Cambridge, 1996.
[34] A.R. Cross, A.W. Segal, The NADPH oxidase of professional phagocytes—prototype of the NOX electron transport chain systems, Biochim. Biophys. Acta 1657 (2004) 1–22.
[35] P. Richard, The rhythm of yeast, FEMS Microbiol. Rev. 27 (2003) 547–557.
[36] Y. Kuramoto, Lecture Notes in Physics, Springer, New York, 1975, pp. 420–422.
[37] S.H. Strogatz, From Kuramoto to Crawford: exploring the onset of synchronization in populations of coupled oscillators, Physica D 143 (2000) 1–20.
[38] R. He, M. Nanamori, H. Sang, H. Yin, M.C. Dinauer, R.D. Ye, Reconstitution of chemotactic peptide-induced nicotinamide adenine dinucleotide phosphate (reduced) oxidase activation in transgenic COS-phox cells, J. Immunol. 173 (2004) 7462–7470.

[39] N. Cheng, R. He, J. Tian, M.C. Dinauer, R.D. Ye, A critical role of protein kinase C delta activation loop phosphorylation in formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of p47(phox) and rapid activation of nicotinamide adenine dinucleotide phosphate oxidase, J. Immunol. 179 (2007) 7720–7728.
[40] S.P. Soltoff, Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta, Trends Pharmacol. Sci. 28 (2007) 453–458.
[41] M. McDermott, M.J. Wakelam, A.J. Morris, Phospholipase D, Biochem. Cell Biol. 82 (2004) 225–253.
[42] A. Nishikimi, H. Fukuhara, W. Su, T. Hongu, S. Takasuga, H. Mihara, Q. Cao, F. Sanematsu, M. Kanai, H. Hasegawa, et al., Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis, Science 324 (2009) 384–387.
[43] M.P. Wymann, P. Kernen, T. Bengtsson, T. Andersson, M. Baggiolini, D.A. Deranleau, Corresponding oscillations in neutrophil shape and filamentous actin content, J. Biol. Chem. 265 (1990) 619–622.
[44] A. Van Keymeulen, K. Wong, Z.A. Knight, C. Govaerts, K.M. Hahn, K.M. Shokat, H.R. Bourne, To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front, J. Cell. Biol. 174 (2006) 437–445.
[45] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (2003) 1704–1709.
[46] J. Xu, F. Wang, A. Van Keymeulen, P. Herzmark, A. Straight, K. Kelly, Y. Takuwa, N. Sugimoto, T. Mitchison, H.R. Bourne, Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils, Cell 114 (2003) 201–214.
[47] M.A. Frohman, T.C. Sung, A.J. Morris, Mammalian phospholipase D structure and regulation, Biochim. Biophys. Acta 1439 (1999) 175–186.
[48] S. Andrews, L.R. Stephens, P.T. Hawkins, PI3K class IB pathway in neutrophils, Sci. STKE 2007 (2007) cm3.
[49] L. Stephens, A. Smrcka, F.T. Cooke, T.R. Jackson, P.C. Sternweis, P.T. Hawkins, A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits, Cell 77 (1994) 83–93.
[50] H. Yano, S. Nakanishi, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nonomura, Y. Matsuda, Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells, J. Biol. Chem. 268 (1993) 25846–25856.
[51] P. Devreotes, C. Janetopoulos, Eukaryotic chemotaxis: distinctions between directional sensing and polarization, J. Biol. Chem. 278 (2003) 20445–20448.
[52] M.B. Hallett, D. Lloyds, The Molecular and Ionic Signaling of Neutrophils, Springer, New York, 1997.
[53] T. Andersson, C. Dahlgren, T. Pozzan, O. Stendahl, P.D. Lew, Characterization of fMet-Leu-Phe receptor-mediated Ca2+ influx across the plasma membrane of human neutrophils, Mol. Pharmacol. 30 (1986) 437–443.
[54] V. Niggli, Signaling to migration in neutrophils: importance of localized pathways, Int. J. Biochem. Cell Biol. 35 (2003) 1619–1638.
[55] D. Fell, Understanding the Control of Metabolism, Portland, London, 1997.
[56] W. Tian, I. Laffafian, S. Dewitt, M.B. Hallett, Exclusion of exogenous phosphatidylinositol-3, 4, 5-trisphosphate from neutrophil-polarizing pseudopodia: stabilization of the uropod and cell polarity, EMBO Rep. 4 (2003) 982–988.
[57] H.R. Petty, Letter of retraction, J. Immunol. 182 (2009) 1770.
[58] J.C. Brasen, A. Lunding, L.F. Olsen, Human myeloperoxidase catalyzes an oscillating peroxidase–oxidase reaction, Arch. Biochem. Biophys. 431 (2004) 55–62.
[59] L.F. Olsen, U. Kummer, A.L. Kindzelskii, H.R. Petty, A model of the oscillatory metabolism of activated neutrophils, Biophys. J. 84 (2003) 69–81.
[60] R.G. Russo, L.A. Liotta, U. Thorgeirsson, R. Brundage, E. Schiffmann, Polymorphonuclear leukocyte migration through human amnion membrane, J. Cell Biol. 91 (1981) 459–467.
[61] P.M. Henson, R.B. Johnston Jr., Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins, J. Clin. Investig. 79 (1987) 669–674.
[62] E.J. Calabrese, Cell migration/chemotaxis: biphasic dose responses, Crit. Rev. Toxicol. 31 (2001) 615–624.
[63] J.D. Violin, J. Zhang, R.Y. Tsien, A.C. Newton, A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C, J. Cell. Biol. 161 (2003) 899–909.
[64] I. Yuli, A. Tomonaga, R. Synderman, Chemoattractant receptor functions in human polymorphonuclear leukocytes are divergently altered by membrane fluidizers, Proc. Natl Acad. Sci. U. S. A. 79 (1982) 5906–5910.
[65] I. Yuli, R. Snyderman, Light scattering by polymorphonuclear leukocytes stimulated to aggregate under various pharmacologic conditions, Blood 64 (1984) 649–655.
[66] B.N. Kholodenko, Cell-signalling dynamics in time and space, Nat. Rev. Mol. Cell. Biol. 7 (2006) 165–176.
[67] V. Chickarmane, B.N. Kholodenko, H.M. Sauro, Oscillatory dynamics arising from competitive inhibition and multisite phosphorylation, J. Theor. Biol. 244 (2007) 68–76.