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Signal processing times in neutrophil activation: dependence on ligand concentration and the relative phase of metabolic oscillations

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Abstract

Intracellular NAD(P)H oscillations exhibited by polarized neutrophils display ≅20 s periods, which are halved to ≅10 s upon stimulation with chemotactic peptides such as FNLPNTL (*N*-formyl-nle-leu-phe-nle-tyr-lys). By monitoring this frequency change, we have measured accurately the time interval between stimulus and metabolic frequency changes. A microscope flow chamber was designed to allow rapid delivery of FNLPNTL to adherent cells. Using fluorescein as a marker, we found delivery to be complete and stable throughout the chamber within ∼400 ms. Peptides were injected into the chamber at concentrations ranging from 10^{-6} to 10^{-9} M. Injections also varied with respect to the relative phase of a cell's NAD(P)H oscillations. The time interval between injection of 10^{-6} M FNLPNTL and the acquisition of ≅10 s period metabolic oscillations was found to be 12.2 ± 3.3 s when injections occurred at the NAD(P)H oscillation peak whereas the lag time was 22.5 ± 4.8 s when coinciding with a trough. At 10^{-8} M FNLPNTL, lag times were found to be 26.1 ± 5.2 and 30.5 ± 7.3 s for injections at NAD(P)H peaks and troughs, respectively. FNLPNTL at 10^{-9} M had no effect on metabolic oscillations, consistent with previous studies. Our experiments show that the kinetics of transmembrane signal processing, in contrast to a simple transmembrane chemical reaction, can depend upon both ligand dose and its temporal relationship with intracellular metabolic oscillations.

Keywords: Neutrophils; NAD(P)H; Activation; Metabolism; Oscillations; Signal processing

1. Introduction

Oscillations are a well-known feature of chemical systems maintained far from equilibrium, such as the Belousov–Zhabotinskii reaction. Living cells, another chemical system maintained far from equilibrium, can also display oscillations [1]. Metabolic oscillations are one type of oscillation found in cells and tissues [1–4]. The concentrations of many metabolites, including NADH, NADPH, ATP, H^+, phosphoenolpyruvate (PEP) and others, oscillate in time [4]. Metabolic oscillations with a period of ∼3 min are produced during glycolysis

Abbreviations: FNLPNTL, *N*-formyl-nle-leu-phe-nle-tyr-lys.

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by allosteric enzymes, especially phosphofructokinase (PFK) [3,4]. PFK's level of activity is dependent upon feedback mechanisms: it is inhibited by its substrate ATP and activated by its product ADP. In addition, NAD(P)H oscillations with periods of ≅10 and/or ≅20 s have been observed in leukocytes, tumor cells, and neural tissues [2,5–10]. The ≅10 s metabolic oscillations appear to be closely linked to the intracellular calcium signaling apparatus as their frequency is affected by low doses of thapsigargin, an endoplasmic reticulum Ca^{+2}-ATPase inhibitor (unpublished observations). However, recent computer simulations have indicated that high amplitude oscillations seen in interferon-γ activated neutrophils are due to the dynamic chemical coupling of the NADPH oxidase and myeloperoxidase [11]. Thus, metabolic oscillations are an emergent cellular property arising from the complex biochemical networks characterized by feedback loops.

We have suggested that certain neutrophil functions are regulated by metabolic oscillations. This hypothesis was initially based upon the correspondence between the periods of NAD(P)H oscillations and the periods of functional oscillations within cells. Early studies demonstrated that a minimum lag time of ≅10 s is observed prior to lamellipodia extension, superoxide production and membrane depolarization in response to activating stimuli (e.g. [12,13]). Many biological response modifiers such as chemotactic factors, cytokines, immune complexes, etc. can alter the frequency (a switch from 20 to 10 s) or amplitude of NAD(P)H oscillations (e.g. [5–10]). Neutrophil physiological responses such as actin polymerization, shape change, reactive oxygen metabolite production, nitric oxide (NO) production, pericellular proteolysis and receptor coupling/uncoupling temporally oscillate with periods of ≅20 or ≅10 s [5–10,14,15], which match the intracellular NAD(P)H oscillation periods of neutrophils [5–10,14,15]. NAD(P)H oscillations are thought to influence NADPH-dependent enzymes such as the NADPH oxidase (superoxide production) and the NO synthase (NO release) [9,10,15]. The extent of superoxide and NO production correlate with the amplitude and frequency of metabolic oscillations and are in phase with NAD(P)H oscillations [6,10]. Thus, we have proposed that metabolic oscillations are tied with many physiologic functions of neutrophils [7,8,15,16].

Intracellular oscillators may provide several chemical and biological advantages to living cells. One important advantage of metabolic oscillations is that they enhance the efficiency of free energy conversion from sugar to ATP [17–19]. Other important advantages of intracellular metabolic and/or signaling oscillations may be: (1) to drive enzymatic and transport pathways away from equilibrium, (2) as a frequency-dependent filter in discriminating among stimuli, (3) systems gain advantages, (4) ability to rapidly change enzyme activity and (5) protect cells from damage mediated by toxic substances (e.g. [15,20–24]). Inasmuch as many intracellular chemical oscillations reach their peaks at different times, their relative 'timing' or phases may coordinate different biochemical reactions. For example, neutrophils produce superoxide anions, which inactive protease inhibitors, immediately prior to the activation of membrane-bound proteases [15]. Since receptor stimulation can be easily manipulated by ligand injection, we explored the temporal or phase relationship between formyl peptide receptor stimulation and NAD(P)H frequency changes. Our results suggest that lag times required for NAD(P)H frequency changes, which correspond to signal processing events within a cell, are influenced by both the ligand concentration and the relative timing of metabolic oscillations and receptor ligation.

2. Materials and methods

2.1. Neutrophil isolation

Whole blood was collected from normal human donors. Neutrophils were isolated using Ficoll Histopaque density gradient centrifugation (Sigma Chem. Co., St. Louis, MO). Isolated neutrophils were collected, re-suspended in HBSS (Hank's balanced salt solution), and washed with HBSS by centrifugation at $400 \times g$. The pelleted cells were re-suspended at 1×10^3 cells/ml and stored on ice.

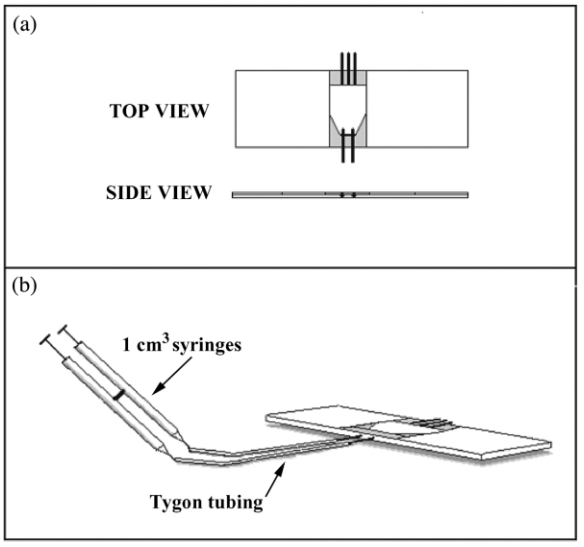

Fig. 1. Diagram of glass injection chamber. (a) The chamber was constructed of glass microscope slides that were assembled with epoxy resin to form the chamber area. Stainless steel tubing was used for the entry and exit ports. (b) Injection syringes connected to chamber. A glass coverslip was attached to the chamber with vacuum grease. The chamber was inverted such that the coverslip formed the bottom of the chamber. A cell suspension was placed into the chamber and warmed on a 37 °C microscope stage prior to experimentation. One cm^3 syringes were loaded with chemotactic factor (FNLPNTL). This chemotactic solution was injected at the peaks or troughs of NAD(P)H sinusoidal waves.

2.2. Chemotactic factor

The chemotactic factor *N*-formyl-nle-leu-phe-nle-tyr-lys (FNLPNTL; Sigma) was used. A stock solution of 10^{-5} M FNLPNTL in sterile HBSS was stored at −20 °C. Dilutions from 10^{-6} M to 10^{-9} M were made from a stock solution prior to experimentation. A 1×10^{-5} M solution of fluorescein (Molecular Probes, Eugene, OR) was used to observe fluid flow in the chamber.

2.3. Chamber

A glass microscope chamber was constructed using a 2.5 cm by 7.5 cm microscope slide, glass coverslips, and 19-gauge (inner diameter 0.69 mm) stainless steel tubing (Fig. 1). The sides parallel to the entry and exit ports were formed by three coverslips stacked vertically and held together with epoxy resin. They were fastened 1 cm apart at the center of the microscope slide. The other sides were formed by 1 cm, 19-gauge stainless steel tubing embedded in epoxy resin (Fig. 1). A coverslip was attached to the chamber using vacuum grease, which gave a total internal volume of 100 μl. The glass chamber was inverted, filled with 100 μl of a neutrophil suspension, and placed on a 37 °C microscopic stage for several min prior to experimentation.

2.4. Microscopy

A Zeiss axiovert fluorescence microscope with a 100 W mercury lamp and a 100× Neofluar objective (Carl Zeiss, New York, NY) was used in these experiments. NAD(P)H autofluorescence microscopy was performed as described [7]. NAD(P)H was detected using a 365DF20 nm excitation filter and 450DF35 nm emission filter and a 405 nm long-pass dichroic mirror. Fluorescence intensities were measured using a photomultiplier tube (Hamamatsu; Bridgewater, NJ) interfaced to a discriminator, amplifier and photon counter (Photochemical Research Associates, Inc.; London, Ont.). The signal was analyzed by Maclab 200 system, using Chart software version 3.7 (AD Instruments, Castle Hill, Australia).

3. Results

The first step in transmembrane signaling is receptor ligation. This is followed by signal transduction via second messengers in the cytosol, an increased metabolic rate, and ultimately heightened levels of certain downstream products. To dissect the relationship between receptor ligation and the frequency doubling of metabolic oscillations, we constructed an injection chamber that could rapidly deliver uniform concentrations of chemoattractant solutions while observing intracellular NAD(P)H oscillations. Fig. 1a shows a diagram of the injection chamber. Prior to experimentation, a glass coverslip was attached to the chamber with vacuum grease. The chamber was inverted, filled with 100 μl of a neutrophil suspension, and connected by rubber tubing to 1 cm^3 syringes containing

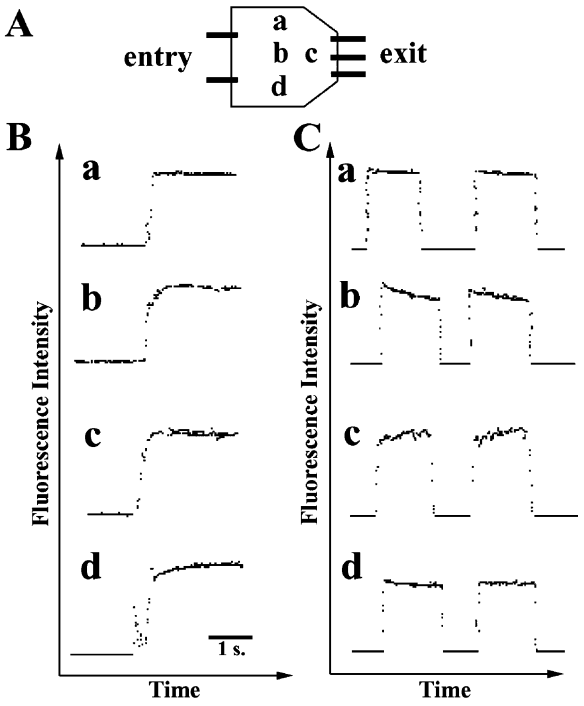

Fig. 2. Fluid distribution in the glass injection chamber. (A) Schematic illustration of the locations (positions a–d) where the fluorescence intensities were monitored. (B) Fluorescein (10^{-5} M) solutions were placed into 1 cm^3 syringes, attached to the chamber, and injected for 1 s. Stair-step increases in fluorescence for each location (a through d) required approximately 400 ms to reach the plateau. These measurements were conducted at the same focal plane used in cellular studies. (C) Fluorescein (10^{-5} M) was injected into the chamber and fluorescence intensities were measured for ~5 s. In these experiments the fluorescence intensity recordings at positions a–d were repeated after a 100 μm horizontal translocation of the objective. No significant differences were observed.

FNLPNTL solutions. The chamber was placed on a heated microscope stage and warmed to 37 °C before experimentation.

To test the injection chamber's fluid exchange properties, we measured the fluorescence intensities at four locations (a through *d* with each 2 mm apart) within the chamber during the injection of 1×10^{-5} M fluorescein (Fig. 2A); these four locations were at the plane of focus for cell imaging (near the coverslip and side-to-side). Location b was the geometric center of the chamber. Fig. 2B shows stair-step increases in fluorescence intensity that required <400 ms to reach the intensity maximum at each of the designated locations. Thus, fluid exchange occurred rapidly throughout the chamber. After monitoring each injection, the objective was moved horizontally 100 μm and the fluorescence intensity was again recorded at each of the four locations (Fig. 2C). The fluorescence intensities after fluorescein injection were identical at all tested positions within the chamber. All tests confirmed that fluid exchange was rapid and uniform throughout the chamber, thereby providing a means to accurately stimulate cells while observing NAD(P)H oscillations.

We hypothesized that cells may require different lengths of time to respond to receptor ligation depending upon the temporal relationship between receptor stimulation and metabolic oscillations. To test this concept, a polarized neutrophil was selected and centered in the microscopic field of view. A pre-stimulation NAD(P)H oscillation was recorded, which invariably displayed a period of ~20 s Subsequently, an FNLPNTL solution was injected at the peak or in other experiments, at the trough of an NAD(P)H oscillation. Only one injection could be performed for each cell. Neutrophils respond to chemotactic factors by doubling the frequency of NAD(P)H oscillations [7]. Frequency doubling lag times were measured from the point of ligand addition to the trough of the first 10 s NAD(P)H oscillation. When 10^{-6} M FNLPNTL was applied at the troughs, frequency doubling occurred in 22.5 ± 4.8 s (Fig. 3a). Fig. 3b shows a representative example of an intracellular NAD(P)H oscillation profile that frequency doubled after an injection of 10^{-6} M FNLPNTL at an oscillation peak. The lag time was 12.2 ± 3.3 s. Cells stimulated at the NAD(P)H oscillation trough with 10^{-8} M FNLPNTL produced frequency doubling after 30.5 ± 7.3 s (Fig. 3c). Similarly, stimulation with 10^{-8} M FNLPNTL at the NAD(P)H peak produced frequency doubling in 26.1 ± 5.2 s (Fig. 3d). However, neutrophils stimulated with 10^{-9} M FNLPNTL showed no changes in NAD(P)H oscillations when stimulated at peaks or troughs (Fig. 3e and f). This is in agreement with previous studies showing that 10^{-9} M *N*-formyl-met-leu-phe (FMLP) had no effect on neu-

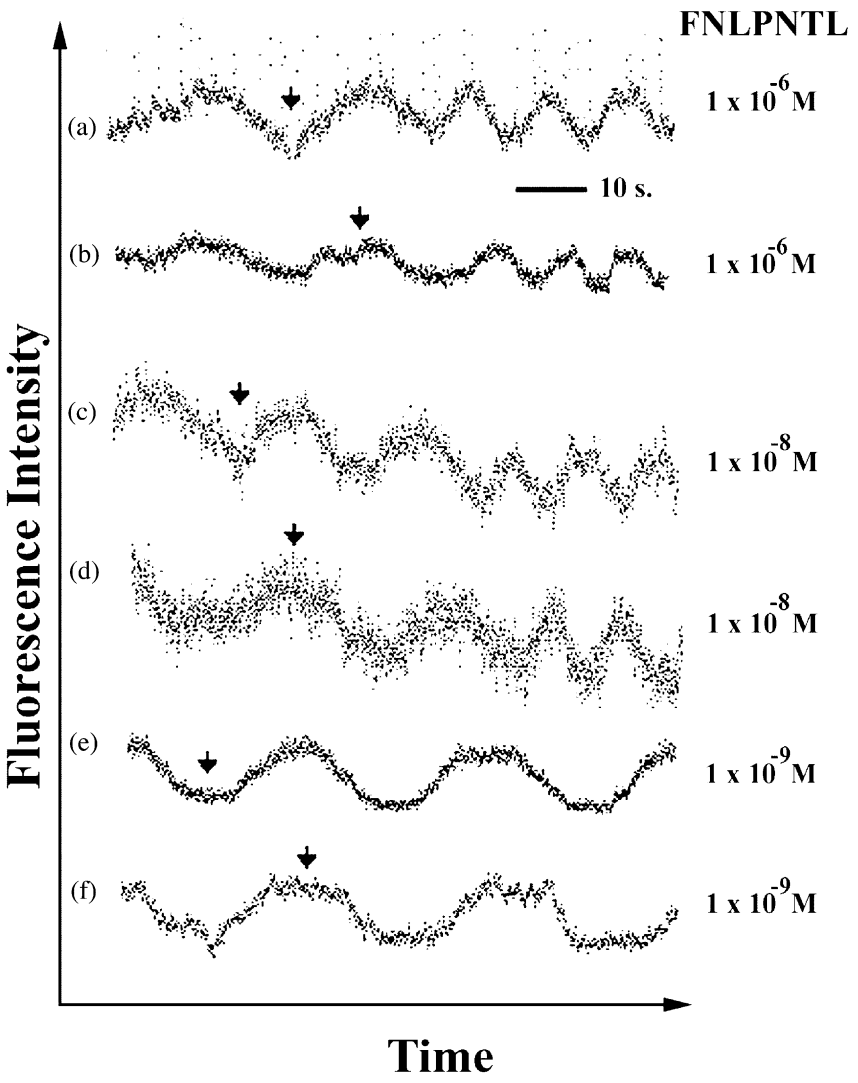

Fig. 3. NAD(P)H oscillations of neutrophils during exposure to various concentrations of FNLPNTL. Arrows indicate time of injection. (a) Injection of 10^{-6} M FNLPNTL at the trough of a 20 s NAD(P)H oscillation. Frequency doubling occurred after 1 oscillation period. (b) Injection of 10^{-6} M FNLPNTL at the peak of a 20 s NAD(P)H oscillation. Frequency doubling occurred after one half of an oscillation period. (c) Injection of 10^{-8} M FNLPNTL at the trough of a 20 s NAD(P)H oscillation. Frequency doubling occurred after 2 oscillation periods. (d) Injection of 10^{-8} M FNLPNTL at the peak of a 20 s NAD(P)H oscillation. Frequency doubling occurred after 1.5 oscillation periods. (e) and (f) Injection of 10^{-9} M FNLPNTL at trough and peak, respectively, of NAD(P)H oscillation. No change in frequency was observed. The number of days upon which these experiments were independently reproduced was: (a) 7, (b) 7, (c) 7, (d) 8, (e) 11 and (f) 11.

trophils [13]. These results suggest that the mechanism of frequency doubling was influenced by the metabolic phase during which ligation occurs, although frequency doubling occurred at all physiologically-relevant concentrations. In addition, as the ligand concentration decreased, the time required for NAD(P)H frequency doubling increased within the concentration range tested.

To better define the concentration-dependence of frequency doubling, we studied a more narrow

Table 1
Lag time for 20 to 10 s sinusoidal NAD(P)H frequency doubling[a]

FNLPNTL concentration	NAD(P)H peak stimulation	NAD(P)H trough stimulation
1×10^{-6} M	12.2±3.3*	22.5±4.8
1×10^{-8} M	26.1±5.2	30.5±7.3
5×10^{-9} M	29.1±6.2	36.1±3.7
3×10^{-9} M	20.8±10.7	38.9±3.1
1×10^{-9} M	No change	No change

[a] S.D. are a reflection of heterogeneity among the 20 s oscillation periods. Injections of 1×10^{-9} M produced no observable changes in frequency. The asterisk indicates significance at a level of 0.01.

range of concentrations. Polarized neutrophils were exposed to 5×10^{-9} and 3×10^{-9} M FNLPNTL solutions using the experimental procedure described above. Injections of 5×10^{-9} M or 3×10^{-9} M FNLPNTL solutions at the peaks of NAD(P)H oscillations produced frequency doubling in 29.1±6.2 and 20.8±10.7 s, respectively. Stimulation at NAD(P)H troughs with 5×10^{-9} M or 3×10^{-9} M FNLPNTL led to frequency doubling in 36.1±3.7 and 38.9±3.1 s, respectively, (Table 1). Average NAD(P)H oscillation frequency doubling lag times are shown in Table 1. The standard deviations represent, in part, the heterogeneity of the 20 s NAD(P)H oscillations. Therefore, decreasing the chemoattractant concentration did not result in progressively longer NAD(P)H frequency doubling lag times.

We next quantified how populations of cells responded to receptor stimulation with respect to metabolic oscillation periods. Fig. 4 shows the percentage of cells demonstrating NAD(P)H oscillation frequency doubling when stimulation occurred at the peak or trough. Stimulation with 10^{-6} and 10^{-8} M FNLPNTL, caused frequency doubling in 100% of the cells tested. As the concentration decreased to 5×10^{-9} M, 81.3% of the cells tested frequency doubled. Frequency doubling occurred in 20% of the cells tested when subjected to 3×10^{-9} M FNLPNTL solutions. Cells exposed to 1×10^{-9} M FNLPNTL at NAD(P)H oscillation peaks or troughs did not frequency double the frequency. This suggests that lag times associated with frequency modulation are binary in nature and may require a critical concentration for frequency doubling to occur (Fig. 4).

4. Discussion

To test for a relationship between known biochemical oscillators and the temporal features of leukocyte responses to chemoattractant stimulation [16], we examined the phase relationship between formyl peptide receptor ligation and the NAD(P)H oscillation changes associated with cell activation. Physiological response lag times have been observed in neutrophils after stimulation with FMLP [12,13]. For example, the study of Gerisch and Keller [12] demonstrated a minimal 10 s signal processing time for cell responses to FMLP. We have also demonstrated that neutrophils optimally turn in response to spatially uniform but temporally decreasing FMLP concentrations when the interval between concentration changes is 10 s [16]. These lag times and intervals match the metabolic oscillation period displayed by FMLP-treated neutrophils. Indeed, our present experiments also demonstrate a minimum lag time of ≅10 s. Moreover, we now show that the minimum lag time is associated with a specific phase of the metabolic

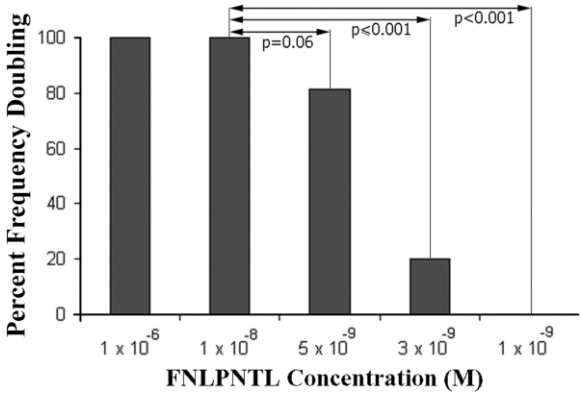

Fig. 4. Percentage of cells that frequency doubled after exposure to different concentrations of FNLPNTL at the peaks and trough of NAD(P)H oscillations. NAD(P)H oscillations were recorded for ~3 min after FNLPNTL injection for 67 cells. These five conditions are compared at the top of the figure, using *P* values to indicate the statistical significance of the experimental conditions identified by the arrows.

clock. Although the mechanism linking receptor ligation with metabolic frequency changes has not yet been established, it seems likely that metabolic oscillations are linked with the processing of signals leading to reactive oxygen metabolite and NO production.

Our results indicate that metabolic activation lag times exhibited during FNLPNTL exposure correlate with the phase of NAD(P)H oscillations during stimulation at certain concentrations within its effective range. Neutrophil receptor stimulation at the NAD(P)H oscillation trough required more time for metabolic changes than stimulation at NAD(P)H oscillation peaks. Since NAD(P)H and ATP oscillate 180° out of phase [4], stimulation at NAD(P)H oscillation peaks occurs at ATP oscillation troughs. Although low ATP and high NAD(P)H levels correlate temporally with rapid signal processing, other biochemical and physiological oscillators are also phase aligned with NAD(P)H oscillators [6]. Thus, we have defined several features of the signal processing events surrounding receptor stimulation. Importantly, signal transduction is not simply a transmembrane chemical reaction, but pre-existing intracellular chemical oscillators also govern signaling kinetics.

One potential mechanism linking receptor ligation with metabolic responses is intracellular calcium. Calcium oscillations, which match the periods of NAD(P)H oscillations, accompany neutrophil migration [6,25]. These calcium oscillations are observed as calcium spikes separated by either $\cong 10$ or $\cong 20$ s. We have shown that the calcium spike, when spatiotemporally resolved using high-speed microscopy, is composed of a periodic calcium wave traveling in the pericytoplasmic region of the cell [26,27]. If the formyl peptide receptor has been ligated, a second calcium channel-dependent calcium wave traveling in the opposite direction is generated at the site of ligand binding when the first calcium wave reaches this point. In other words, the second calcium 'activation' signal only occurs during a calcium spike. Furthermore, calcium signals appear to be an essential step in neutrophil activation (e.g. [28]). It is possible to account for the phase-dependence of FNLPNTL-mediated metabolic activation by the relative timing of the calcium signal. Since the intracellular calcium spike occurs just after the NAD(P)H peak [6], receptor ligation will take place just prior to the calcium peak, thus giving rise to the shortest lag time preceding the new calcium signal. Similarly, if stimulation occurs just after a calcium spike, it will take longer for another calcium spike to return. In addition, several previous investigators have suggested a relationship between the NAD(P)H and calcium oscillators [29,30]. Thus, we tentatively suggest that the timing of receptor stimulation with respect to calcium spikes may account for the length of the signal processing time in neutrophils.

Our results also demonstrate that time required for NAD(P)H frequency changes are concentration-dependent. Stimulation with 10^{-6} M FNLPNTL at the peak and trough initiated lag times in one-half and one NAD(P)H oscillation period (Fig. 3). Yet, lowering the stimulating chemoattractant concentration 100-fold to 10^{-8} M FNLPNTL increased the lag time to 1.5 and 2 NAD(P)H oscillations for peak and trough stimulations, respectively, (Fig. 3). This suggests several characteristics of frequency changes. The increase in NAD(P)H frequency is an all or none event and is dependent upon ligand concentration. Since the K_d for FNLPNTL is ≈ 2.2 nM [31], $>50\%$ receptor occupancy may be needed to stimulate the formation of $\cong 10$ s NAD(P)H oscillations. The increased NAD(P)H response lag times during stimulation with 10^{-8} M FMLP may indicate that signaling intermediates must reach concentration thresholds to respond, which is consistent with wave ignition [26,32]. Thus, lower chemoattractant concentrations may require more time to accumulate intermediates to reach a critical concentration that initiates the changes in NAD(P)H oscillations. An example of such thresholds is the ignition of signaling during cell adherence. During neutrophil adherence, NAD(P)H accumulates at the adherence site until a critical concentration is reached which allows NAD(P)H wave propagation throughout the cell's cytoplasm [32].

Our findings may be relevant to the mechanism of cell chemotaxis. For example, the time lags noted here may be linked with temporal models of chemotactic factor detection and cell migration [33,34]. In other words, cells are periodically

insensitive to a chemotactic factor, which could allow a cell to make comparisons between a factor's present and past concentrations. It is possible that the recently described dynamic uncoupling of the phospholipase C/inositol trisphosphate pathway [35], which also occurs with the frequency described here, is also linked with cell metabolic changes. The study of cellular oscillators and their integration with physiological processes will contribute fundamental insights into our understanding of how intracellular chemistry affects cell function.

Acknowledgments

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