

# Direct measurement of oscillatory generation of superoxide anions by single phagocytes

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**Abstract** Phagocytic cells such as neutrophils generate superoxide anions ( $O_2^-$ ) within phagocytic vacuoles for killing and digesting microorganisms. Here we report the simultaneous observation of morphological changes and  $O_2^-$  generation in single phagocytic cells during phagocytosis. Point stimulation of a cell by contact with an opsonized microelectrode at the cell surface induced significant deformation to engulf the electrode, and also induced the  $O_2^-$  generation which was measured by the electrode. Periodic fluctuations in the magnitude of the  $O_2^-$  generation were observed in the time course. These oscillations may be caused by metabolic regulation of the formation of NADPH, which is the substrate for the  $O_2^-$  generation.

**Key words:** Phagocytosis; Superoxide generation; Biological oscillation; NADPH oxidase; HL-60 cell

## 1. Introduction

'Professional' phagocytes play crucial roles in host defense against pathogens. During phagocytosis, these cells exhibit the 'respiratory burst', i.e. intense consumption of  $O_2$  to produce active oxygen species within phagocytic vacuoles for killing and digesting the engulfed microbes. These species arise from superoxide anions ( $O_2^-$ ) which are generated at the extracellular surface of vacuolar membranes in response to contact with opsonized microbes [1–4]. Large numbers of biochemical studies have been performed on mechanisms of the  $O_2^-$  generation and its regulation, and revealed that the reaction is catalyzed by an enzyme system 'NADPH oxidase', which consists of several cytosolic proteins and a membrane bound *b*-type cytochrome (cyt *b*<sub>558</sub>). Cyt *b*<sub>558</sub> catalyzes one electron reduction of  $O_2$  [5–7], and the cytosolic proteins are involved in the activation of cyt *b*<sub>558</sub> [1–3].

Phagocytic functions such as phagocytosis and the  $O_2^-$  generation are induced via a variety of intracellular signal transduction routes, and have close relevance to each other. However, information on the  $O_2^-$  generating activity obtained by conventional spectroscopic or electrochemical methods is averaged over a mass of cells, and is hardly correlated with other activities of individual cells. In this work, we investigated the phagocytic functions in individual cells by a microelectrochemical technique [8–10] with subpicoampere sensitivity. We stimulated

single phagocytic cells by contact with a microelectrode coated with IgG or phorbol myristate acetate (PMA), and measured the  $O_2^-$  generation under observation of the morphological changes. We found oscillations in the  $O_2^-$  generation from individual cells, and discussed mechanisms of the oscillations in terms of regulatory factors of the reaction.

## 2. Materials and methods

A cylinder-shaped microelectrode was fabricated by mounting a carbon fiber with a diameter of about 10  $\mu$ m into a glass capillary tube and fixed with epoxy resin [9,10]. Before the experiments, the tip of the electrode was polished to obtain a clean beveled surface at an angle of approximately 45°. It was dipped for 15 min into phosphate-buffered saline (PBS) solution containing 80 mg/ml of human IgG at a temperature of 37°C, and was dried at room temperature. This procedure was repeated three times. IgG was purified from human blood with Protein A Sepharose-4B (Pharmacia). Otherwise, the electrode was coated with PMA by dipping into ethanol solution containing 80 mM PMA (Sigma) in place of the IgG-containing PBS solution.

A human promyelocytic leukemia cell line, HL-60 [11,12] was differentiated to induce phagocytic functions by retinoic acid, and was used for experiments as a model of granulocytes. HL-60 cells maintained in RPMI 1640 medium (Nissui Seiyaku, Japan) supplemented with 10% fetal calf serum (Gibco) were plated onto a collagen (Koken Inc., Japan)-coated glass coverslip in the culture medium which contained 1  $\mu$ M retinoic acid (Sigma). After incubation for 3 or 4 days, more than 90% of the cells were induced to differentiate, and were fixed on the collagen surface. After washing the cells with PBS solution containing 5 mM  $Ca^{2+}$ , one of these cells was stimulated by contact with an opsonized or PMA-coated microelectrode. The  $O_2^-$  generation was measured by a 2-electrode system with Fuso Seisakusho Model 325, which was poised at a potential of +0.1 V vs. Ag/AgCl. The experiment was monitored by an Olympus IMT-2 microscope equipped with Hamamatsu C2400 video camera.

## 3. Results and discussion

HL-60 cells were fixed on a collagen surface during the induction to differentiate by the incubation with retinoic acid. One of these cells was stimulated by contact with an opsonized electrode. Typically, a stimulated cell showed shape changes as shown in Fig. 1A. First, the cell adhered to the tip of the microelectrode (Fig. 1Aa,b), then extended the lamellipodium and surrounded the tip (c,d). The cell further deformed (e–h), and finally returned to a spherical shape (i). The simultaneously measured time course of  $O_2^-$  generation is shown in Fig. 1B. When the cell began to change its shape, about 4 min after the contact with the electrode, the first rise of the oxidation current was observed. Thereafter, the current gradually increased to a maximum at around 12 min, and gradually decreased near the baseline level after 30 min. Interestingly, small, repeated increases and decreases in the current over-

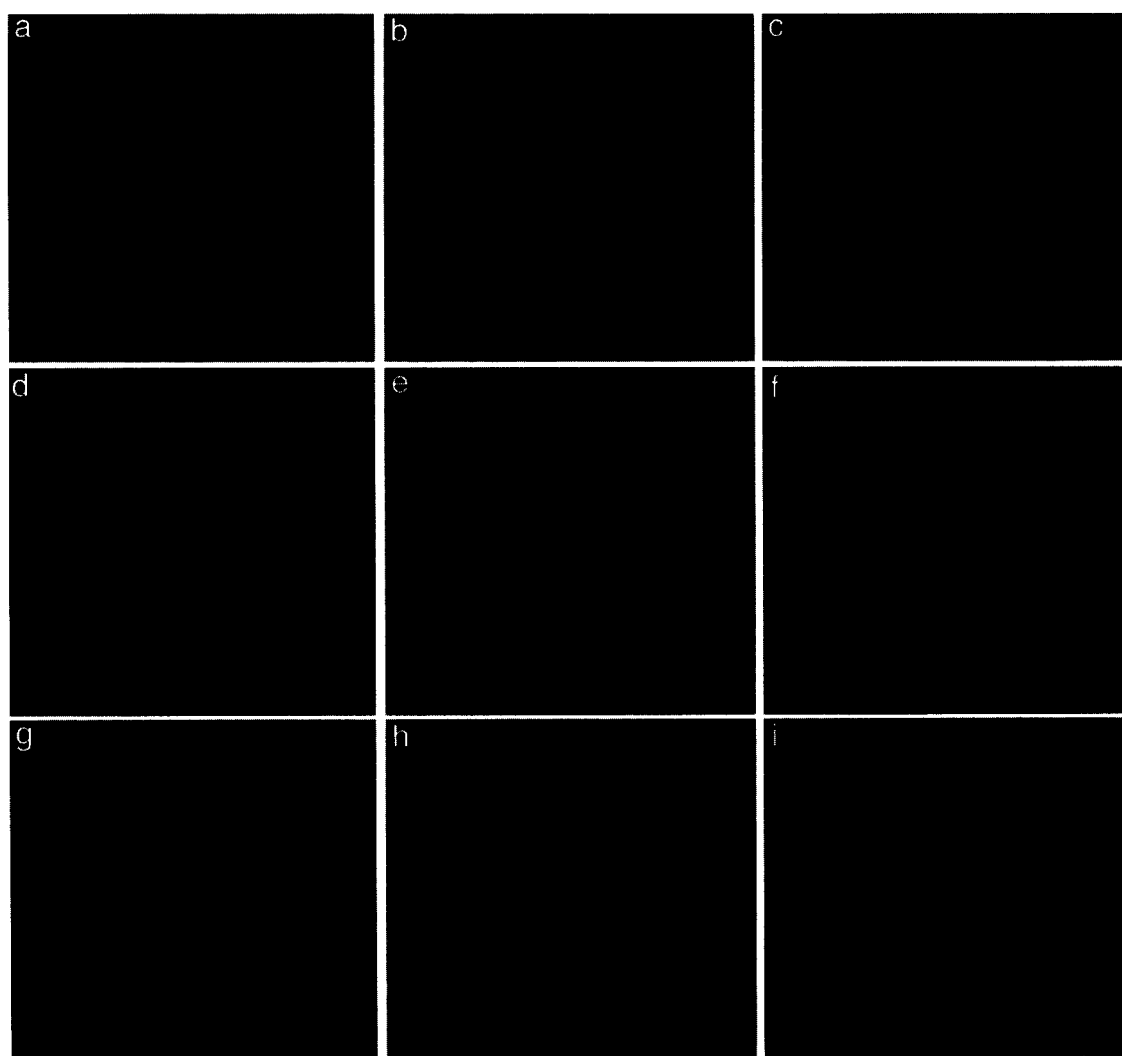
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lapped on the entire time course. These oscillations with an approximate period of 1 min were significant in the first several turns (see also Fig. 1C). The addition of superoxide dismutase decreased the current to the background level completely, confirming that the anodic current arose from the oxidation of  $O_2^-$  (Fig. 1B). In another case as shown in Fig. 2A, which shows an unusually larger current maximum, the oscillations were also observed during the early time course, and subsequently attenuated and disappeared. Apparent oscillations were observed in five of nine independent measurements including Fig. 2A.

In contrast to the cases with the opsonized electrode, the cells stimulated with a microelectrode coated with PMA did not change their shape to engulf the electrode. The  $O_2^-$  generation induced by the PMA electrode always started with shorter delay of about 2 min after the contact, and continued for more than 1 h, longer than that induced by the opsonized electrode (Fig. 2B). In the cases with the PMA electrode, the oscillations were not so clear as in those with the opsonized

electrode. When the cells were contacted with an uncoated microelectrode, neither morphological changes nor  $O_2^-$  generation were observed.

Average profiles of the  $O_2^-$  generation measured with IgG and PMA microelectrodes are summarized in Table 1. These differences between the results with IgG and PMA seem to be due to differences in the activation process of NADPH oxidase and in the following regulatory process [1–3]. In the cases with IgG electrodes, single cells produced  $O_2^-$  only at the surface that contacted with the electrode, which was confirmed by no detection of current at other sites of the cell surface. On the other hand, cells stimulated by PMA electrodes produced  $O_2^-$  not only at the contacted sites but the whole surfaces. Thus, the data on maximum rate and total amounts of  $O_2^-$  should be magnified by 10 times, approximate ratio of the whole surface area to the contacted area, to estimate the values per single whole cell, in the cases with the PMA electrodes. This revision gives good agreement with values determined by conventional methods with a mass of cells; by a Clark-type



**A** Fig. 1. Point stimulation of a phagocytic cell by contact with an opsonized microelectrode. (A) Morphological changes of the stimulated cell: a–i indicate pictures taken at 1, 3, 7, 11, 15, 19, 23, 27, 32 min after contact with the opsonized microelectrode. (B) Current–time profile of the  $O_2^-$  generation from the same cell as in A. At time 0, the cell was contacted with the opsonized microelectrode. The arrow indicates the addition of superoxide dismutase to a final concentration of 50  $\mu$ g/ml. (C) The first derivative of the current profile in the early time range of (B). Asterisks indicate apparent waves of the oscillations with a period of  $67 \pm 11$  s. For experimental details, refer to section 2.

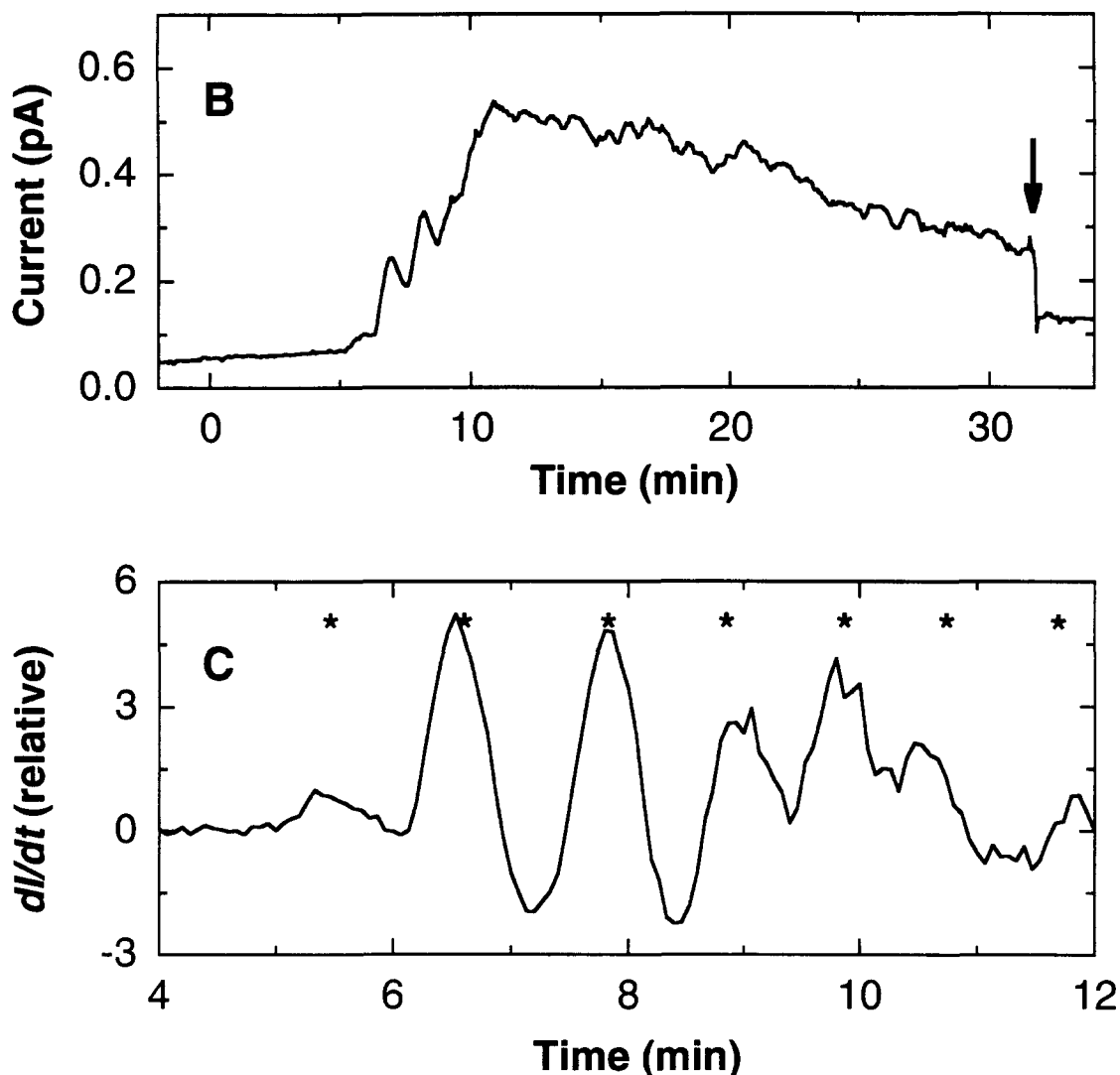


Fig. 1. (continued).

oxygen electrode and by a spectroscopic method with cytochrome *c* [10].

The observed oscillations of  $O_2^-$  generation (Figs. 1B and 2A) possibly occur through a cooperative, synchronous process that is involved in the activation of NADPH oxidase, such as changes in the intracellular concentration of calcium ions

Table 1  
Average profiles of  $O_2^-$  generation by single cells

Stimulant	Maximum rate (fmol $O_2^- \cdot \text{min}^{-1}$ )	Total amount of $O_2^-$ (fmol)
IgG <sup>a</sup>	$1.2 \pm 0.4$	$15 \pm 5$
PMA <sup>b</sup>	$0.4 \pm 0.1^c$	$30 \pm 7^c$

<sup>a,b</sup>Mean values  $\pm$  S.D. based on measurements of 9 and 8 measurements, respectively.

<sup>c</sup>Data should be magnified by 10 times to estimate the values per single whole cell (see text).

( $[Ca^{2+}]_i$ ) [13,14]: the oxidases within single cells may periodically exchange between inactive and active states during the respiratory burst, and this exchange may be controlled by or synchronized with  $[Ca^{2+}]_i$ . This is supported by the contrastive results with PMA, in which  $O_2^-$  oscillations were not clear, since PMA can activate the  $O_2^-$  generation by bypassing the  $Ca^{2+}$  routes [1,2]. Actually, in separate calcium-imaging experiments using fluorescent calcium-sensitive dyes, we observed that  $[Ca^{2+}]_i$  of a specific area near the opsonized electrode contact site oscillated with a period similar to that of the  $O_2^-$  oscillations (Isogai, Y., Tanaka, K., Shinoda, Y. and Suematsu, M., unpublished data). However, we have obtained no direct evidence that correlates the  $O_2^-$  generation with the changes in  $[Ca^{2+}]_i$ . A more plausible explanation is metabolic control of the formation of NADPH, which is the electron donor for the  $O_2^-$  generation. NADPH is produced by oxidation of glucose phosphate in the pentose phosphate pathway, in which the dehydrogenation of glucose phosphate is rate-limiting and serves as the control site

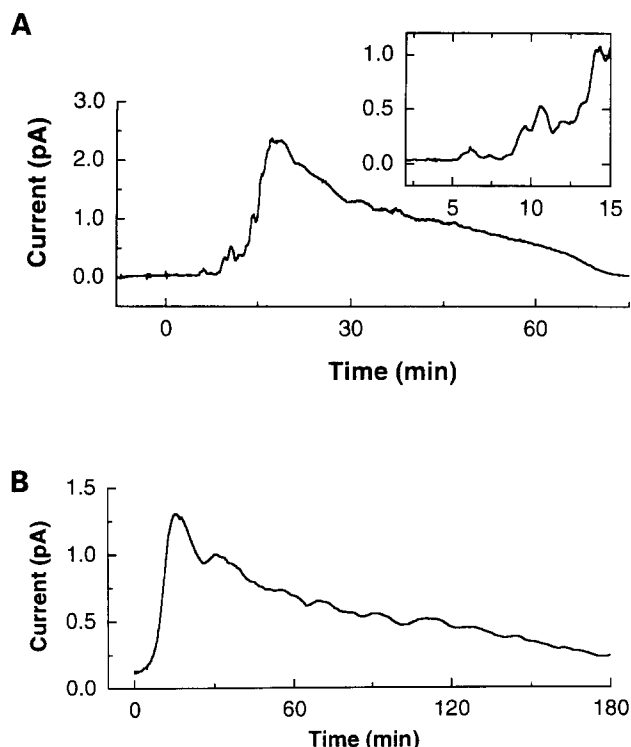


Fig. 2. Current–time profiles of the  $O_2^-$  generation from single cells. Each cell was stimulated by an opsonized microelectrode (A) and PMA-coated microelectrode (B). Inset of A shows an enlarged part of the early time range of the profile. Experiments were carried out as those in Fig. 1.

[15]. The most important regulatory factor is the ratio of  $NADP^+$  to  $NADPH$ , which is maintained at a low level under the dormant physiological conditions. In the respiratory burst, the ratio is always governed by the balance of formation and consumption of  $NADPH$ . Consequently, the change in the level of activated  $NADPH$  oxidase should cause fluctuations in  $NADPH$  concentration and, hence, the observed oscillations in  $O_2^-$  generation.

In this study, physiological function of the  $O_2^-$  oscillations in phagocytes is not clear. However, in the light of the putative roles of noxious small molecules such as nitric oxide and carbon monoxide as neural messengers [16,17], the present finding and

the proposed mechanism of the oscillations suggest similar manners in the generation of these molecules, which also requires  $NADPH$  as a substrate.

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