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# Phorbol myristate acetate potentiates superoxide release and membrane depolarization without affecting an increase in cytoplasmic free calcium in human granulocytes stimulated by the chemotactic peptide, lectins and the calcium ionophore

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**We investigated the inter-relationships of superoxide ($O_2^-$) release, membrane depolarization and an increase in cytoplasmic free $Ca^{2+}$, $[Ca^{2+}]_i$, in human granulocytes stimulated by various agonists. When concanavalin A or the $Ca^{2+}$ ionophore ionomycin was used as stimulus, an increase in $[Ca^{2+}]_i$ clearly preceded the onset of membrane depolarization, which was followed by $O_2^-$ release. On the other hand, when *N*-formylmethionylleucylphenylalanine or wheat-germ agglutinin was used as stimulus, no demonstrable lag was seen in any of the responses. $O_2^-$ release and membrane depolarization stimulated by all these agonists were markedly potentiated in parallel by pretreatment of cells with a low concentration of phorbol myristate acetate (0.25 ng/ml), whereas an increase in $[Ca^{2+}]_i$ was not affected or minimally potentiated. The lag time between addition of the stimulus (concanavalin A or ionomycin) and onset of membrane depolarization or $O_2^-$ release was significantly reduced by pretreatment of cells with phorbol myristate acetate, whereas the lag time between addition of concanavalin A and onset of the increase in $[Ca^{2+}]_i$ was not affected. The dose-response curves for triggering of $O_2^-$ release and membrane depolarization by each of receptor-mediated agonists in phorbol myristate acetate-pretreated or control cells were identical. These findings suggest that; (a) an increase in $[Ca^{2+}]_i$ stimulates membrane depolarization indirectly; (b) a low concentration of phorbol myristate acetate potentiates membrane depolarization and $O_2^-$ release by acting primarily at the post-receptor level, in particular, at the level distal to an increase in $[Ca^{2+}]_i$, but not by augmenting an increase in $[Ca^{2+}]_i$; and (c) the system provoking membrane depolarization and the system activating NADPH oxidase share a common pathway, which may be susceptible to a low concentration of phorbol myristate acetate.**

Abbreviations: Con A, concanavalin A; fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; di-O-$C_5$(3), 3,3′-dipentyloxacarbocyanine; DMSO, dimethylsulfoxide; HBBS, Hanks' balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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## Introduction

Sequential stimulation of human granulocytes with combinations of heterologous stimuli, including chemotactic factors, lectins, calcium ionophores and phorbol esters, results in an enhanced release of superoxide ($O_2^-$) by the second stimulus. This phenomenon is designated as 'activation' or 'priming' by the first stimulus [1–4]. Oxygen metabolites such as $O_2^-$, hydrogen peroxide and hydroxyl radical play an important role in microbial killing by granulocytes [5]. Thus, it is likely that the priming of the respiratory burst, which may also occur in vivo [6], may play an important role in host defenses against microorganisms [7]. It has been recently reported that the enhanced activity of the respiratory burst in primed granulocytes is attributed to an increase in the amount of active $O_2^-$-producing enzyme (NADPH oxidase) rather than alterations in the enzyme itself or activation of a different enzyme [8,9]. This raises the possibility that the first priming stimulus modifies the activating system of NADPH oxidase and renders it more sensitive to the second stimulus. In regard to the activating system, membrane potential changes and an increase in cytoplasmic free $Ca^{2+}$, $[Ca^{2+}]_i$, are proposed to be closely associated with activation of the oxidative metabolism [10–16]. Thus, one possible mechanism for priming is that the priming stimulus may enhance $O_2^-$ release by potentiating the changes in membrane potential, the increase in $[Ca^{2+}]_i$, or both. In this paper, we investigated the effect of phorbol myristate acetate, a potent activator of protein kinase C [17], on $O_2^-$ release, membrane depolarization and an increase in $[Ca^{2+}]_i$ in human granulocytes stimulated by various agonists, including concanavalin A (Con A), wheat-germ agglutinin, fMet-Leu-Phe and the calcium ionophore, ionomycin. The data presented here show that an increase in $[Ca^{2+}]_i$ precedes the onset of membrane depolarization, which is followed by $O_2^-$ release, and that a low concentration of phorbol myristate acetate potentiates membrane depolarization and $O_2^-$ release without affecting an increase in $[Ca^{2+}]_i$ stimulated by various agonists.

## Materials and Methods

*Reagents.* Cytochrome *c* type III, superoxide dismutase, phorbol myristate acetate, Con A, fMet-Leu-Phe and quin2 acetoxymethyl ester (quin2/AM) were purchased from Sigma, St. Louis, MO; wheat germ agglutinin from E.Y. Laboratories, San Mateo, CA; ionomycin from Calbiochem, La Jolla, CA; di-O-$C_5$(3) (3,3′-dipentyloxacarbocyanine) from Japanese Research Institute for Photosensitizing Dyes, Okayama; Conray from Mallinckrodt Inc., St. Louis, MO; Ficoll from Pharmacia Fine Chemicals, Piscataway, NJ; and dextran from Nakarai Chemical Co., Kyoto. Phorbol myristate acetate, fMet-Leu-Phe, ionomycin and quin2/AM were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the reaction mixture was under 0.25%. Stock solutions of di-O-$C_5$(3) were made in ethanol and kept in the dark at 4° C.

*Preparation of cells.* Granulocytes from healthy adult donors were prepared as described [13], using dextran sedimentation and centrifugation with Conray-Ficoll. Contaminated erythrocytes in granulocyte fractions were removed by hypotonic lysis. Granulocyte fractions were suspended in Hanks' balanced salt solutions (HBSS) (Nissui Seiyaku Co., Tokyo) and contained more than 98% granulocytes.

*Determination of $O_2^-$ release.* $O_2^-$ was assayed spectrophotometrically by the reduction of ferricytochrome *c*, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double-wavelength spectrophotometer; Hitachi, Tokyo), equipped with thermostatted cuvette holder as described [13]. The cell suspension in HBSS was added to a 1 ml cuvette containing 110 μM ferricytochrome *c* to obtain final volume of 0.9975–0.9875 ml. Final cell concentration was $1 \cdot 10^6$ cells/ml. The reaction mixture in a cuvette was preincubated at 37° C for 3 min. The cuvette was put in a thermostatted cuvette holder (37° C) of a spectrophotometer and the reduction of cytochrome *c* was measured at 550 nm with a reference wavelength at 540 nm. Various stimulating agents (2.5–10 μl) were added to the reaction

mixture in cuvettes to obtain final volume of 1 ml and the desired concentrations of these agents, while the time-course of cytochrome *c* reduction (the absorbance change at 550–540 nm) was followed on the recorder. The release of $O_2^-$ was calculated from cytochrome *c* reduced for 5 min after the addition of fMet-Leu-Phe, and from the initial linear portion of the cytochrome *c* reduction for phorbol myristate acetate, Con A or wheat-germ agglutinin.

*Determination of membrane potential changes.* Changes in the transmembrane potential were measured by using the fluorescent carbocyanine dye, di-O-$C_5$(3) as described [13]. The fluorescence was measured with a Hitachi MPF-4 fluorescence spectrophotometer equipped with thermostatted cuvette holder (37 °C). The cell suspension in HBSS was added to a 3 ml cuvette containing 0.25 μM di-O-$C_5$(3) to obtain a final volume of 3 ml. Final cell concentration was $1 \cdot 10^6$ cells/ml. The cells were equilibrated with the dye for 10 min at 37 °C before stimuli were added. The cell suspension was maintained by means of a magnetic flea and stirrer. The excitation and emission wavelength were set at 460 nm and 512 nm, respectively. The magnitude of membrane potential changes was calculated from the maximal change after the addition of fMet-Leu-Phe, and from the initial linear portion for phorbol myristate acetate, Con A or wheat-germ agglutinin, and was expressed as the percentage of the resting level as described [18].

*Determination of cytoplasmic free $Ca^{2+}$.* Cytoplasmic free $Ca^{2+}$, $[Ca^{2+}]_i$, was measured by using the fluorescent calcium indicator, quin2, as described [19]. Cells ($5 \cdot 10^7$ cells/ml) suspended in HBSS were equilibrated at 37 °C for 5 min. Quin2/AM (15 μM) was added and the cells were incubated at 37 °C for 15 min in a shaking water bath. The cells were diluted to $1 \cdot 10^7$ cells/ml with warm HBSS, and incubated for another 40 min at 37 °C. After loading, the cells were washed twice, suspended in Hepes buffer (145 mM NaCl/5 mM KCl/1 mM $Na_2HPO_4$/1 mM $CaCl_2$/5 mM glucose/10 mM Hepes (pH 7.4)), and kept at room temperature until used. The fluorescence was measured with a Hitachi MPF-4 fluorescence spectrophotometer, equipped with thermostatted cuvette holder (37 °C). The cell suspension in Hepes buffer was added to a 3 ml cuvette to obtain final volume of 2 ml. Final cell concentration was $5 \cdot 10^6$ cells/ml. The cell suspension was maintained by means of a magnetic flea and stirrer. The excitation and emission wavelength were set at 339 nm and 492 nm, respectively.

*Statistical analysis.* The Student *t*-test was used to determine statistical significance.

## Results

*Effect of phorbol myristate acetate on $O_2^-$ release and membrane potential changes stimulated by Con A*

Phorbol myristate acetate is a highly potent stimulus for inducing $O_2^-$ release and membrane potential changes (depolarization) in human granulocytes [13]. As shown in Fig. 1, phorbol myristate acetate induced $O_2^-$ release and membrane depolarization in a dose-dependent manner, and the dose-response curves for triggering of $O_2^-$ release and membrane depolarization were identical. The stimulation of both functions by phorbol myristate acetate was marginal at 0.25 ng/ml, and negligible at 0.1 ng/ml. However, phorbol myristate acetate was able to prime human granulocytes, even at these low concentrations. The pretreatment of cells with phorbol myristate acetate (0.1 or 0.25 ng/ml) for 5 min at 37 °C potentiated $O_2^-$ release and membrane depolarization stimulated by Con A, whereas Con A by itself was a weak stimulus for inducing both responses when

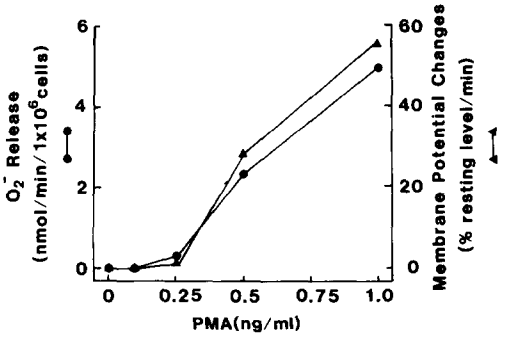


Fig. 1. $O_2^-$ release and membrane potential changes in granulocytes as a function of the concentration of phorbol myristate acetate used as stimulus. Data are expressed as means of three experiments, each done in triplicate. PMA, phorbol myristate acetate.

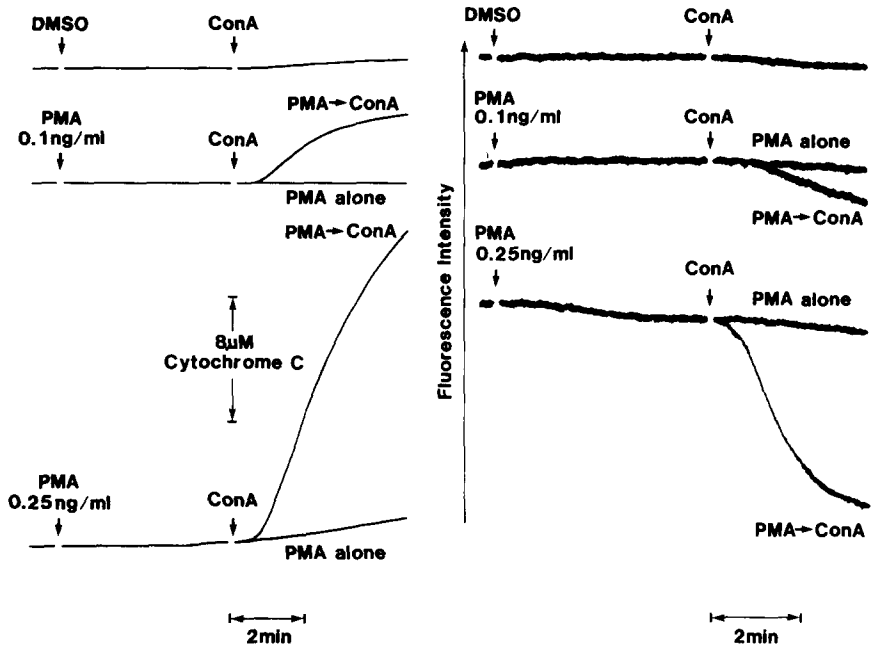


Fig. 2. Time-courses of $O_2^-$ release (left panel) and membrane potential changes (right panel) in granulocytes stimulated by the sequential addition of phorbol myristate acetate and Con A. Phorbol myristate acetate (0.1 or 0.25 ng/ml) was added initially and 5 min thereafter Con A (100 μg/ml) was added. The same concentration of DMSO was added to the control cells. Final cell concentration was $1 \cdot 10^6$ cells/ml. PMA, phorbol myristate acetate.

applied alone. The representative time-courses of $O_2^-$ release and membrane potential changes stimulated by the sequential addition of phorbol myristate acetate and Con A are shown in Fig. 2. The initial rate as well as the total amount of $O_2^-$ release, and the initial rate as well as the maximal change in membrane potential were markedly potentiated. The priming effect was dependent on the concentration of phorbol myristate acetate, and was more remarkable when 0.25 ng/ml phorbol myristate acetate was used. In order to minimize the responses provoked by phorbol myristate acetate itself and to maximize the priming effect of phorbol myristate acetate, we used 0.25 ng/ml phorbol myristate acetate in the following experiments. As shown in Fig. 3, the enhancement of $O_2^-$ release and membrane depolarization by phorbol myristate acetate was observed at all concentrations of Con A used as stimulus (up to 100 μg/ml). The dose-response curves for triggering of $O_2^-$ release and membrane depolarization by Con A in phorbol myristate acetate-pretreated cells were identical. The lag time between addition of Con A (100 μg/ml) and onset of membrane depolarization in phorbol myristate acetate-pretreated cells was $18 \pm 10$ s ($n = 3$), and significantly ($P < 0.01$) reduced as compared with

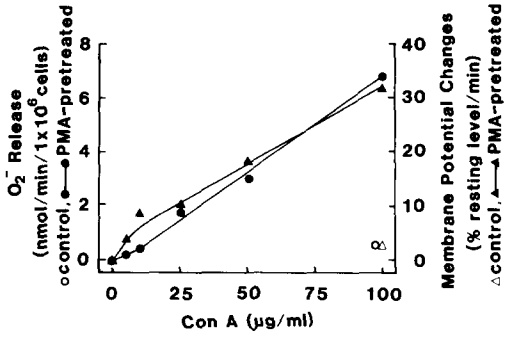


Fig. 3. Effect of phorbol myristate acetate on $O_2^-$ release and membrane potential changes stimulated by Con A. The cells ($1 \times 10^6$/ml) were preincubated with or without phorbol myristate acetate (0.25 ng/ml) for 5 min at 37 °C, and stimulated by the indicated concentrations of Con A. The values of $O_2^-$ release and membrane potential changes stimulated by phorbol myristate acetate alone were subtracted from those stimulated by Con A (see Fig. 2). Data are expressed as means of 3–5 experiments, each done in triplicate. PMA, phorbol myristate acetate.

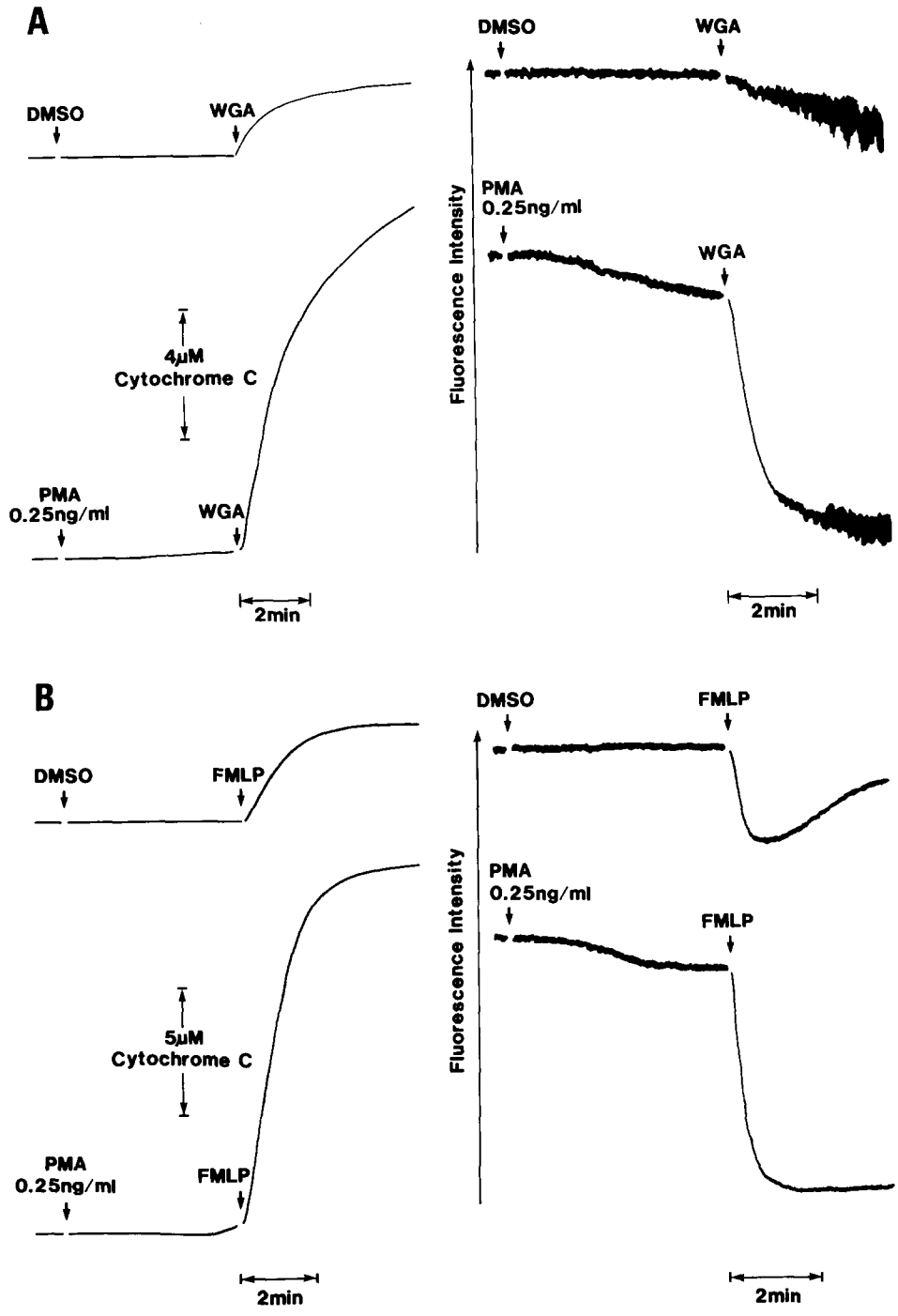


Fig. 4. Time courses of $O_2^-$ release (left panel) and membrane potential changes (right panel) in granulocytes stimulated by the sequential addition of phorbol myristate acetate and wheat germ agglutinin (A), or fMet-Leu-Phe (B). Phorbol myristate acetate (0.25 ng/ml) was added initially and 5 min thereafter wheat-germ agglutinin (50 μg/ml) or fMet-Leu-Phe ($10^{-7}$ M) was added. The same concentration of DMSO was added to the control cells. Final cell concentration was $1 \cdot 10^6$ cells/ml. FMLP, fMet-Leu-Phe. PMA, phorbol myristate acetate; WGA, wheat-germ agglutinin.

that in control cells ($78 \pm 13$ s, $n = 3$). The lag time between addition of Con A and onset of $O_2^-$ release in phorbol myristate acetate-pretreated cells was $50 \pm 3$ s ($n = 3$), and significantly ($P < 0.01$) reduced as compared with that in control cells ($73 \pm 4$ s, $n = 3$). Thus, Con A-induced changes in membrane potential clearly preceded detectable release of $O_2^-$ when phorbol myristate acetate-pretreated cells were used. However, this sequence was not clear when control cells were used, because of the low activity of $O_2^-$ release and membrane depolarization.

*Effect of phorbol myristate acetate on $O_2^-$ release and membrane potential changes stimulated by wheat-germ agglutinin or fMet-Leu-Phe*

We have previously reported that wheat-germ agglutinin is a potent stimulus for inducing $O_2^-$ release in human granulocytes (Fig. 4A) [1,2]. As shown in Fig. 4A, wheat-germ agglutinin also induced membrane potential changes (depolarization) in human granulocytes. No demonstrable lag time was detected in wheat-germ-agglutinin-induced $O_2^-$ release and membrane depolarization. Wheat-germ agglutinin induced the transient release of $O_2^-$, which almost subsided within 5 min. The time-course of $O_2^-$ release stimulated by wheat germ agglutinin was similar to that stimulated by fMet-Leu-Phe. On the other hand, the time-course of membrane depolarization stimulated by wheat-germ agglutinin was different from that stimulated by fMet-Leu-Phe. Wheat-germ agglutinin induced a gradual depolarization, whereas fMet-Leu-Phe induced a rapid depolarization followed by a partial repolarization (Fig. 4). Wheat-germ agglutinin caused remarkable cell aggregation, which explained the remarkable light scattering shown in Fig. 4A. The pretreatment of cells with phorbol myristate acetate (0.25 ng/ml) for 5 min at 37 °C markedly potentiated $O_2^-$ release and membrane depolarization stimulated by wheat-germ agglutinin. The initial rate as well as the total amount of $O_2^-$ release, and the initial rate as well as the maximal change in membrane potential were markedly potentiated. As shown in Fig. 5A, the enhancement of $O_2^-$ release and membrane depolarization by phorbol myristate acetate were observed at all concentrations of wheat-germ agglutinin used as stimulus (up to 50 μg/ml). The

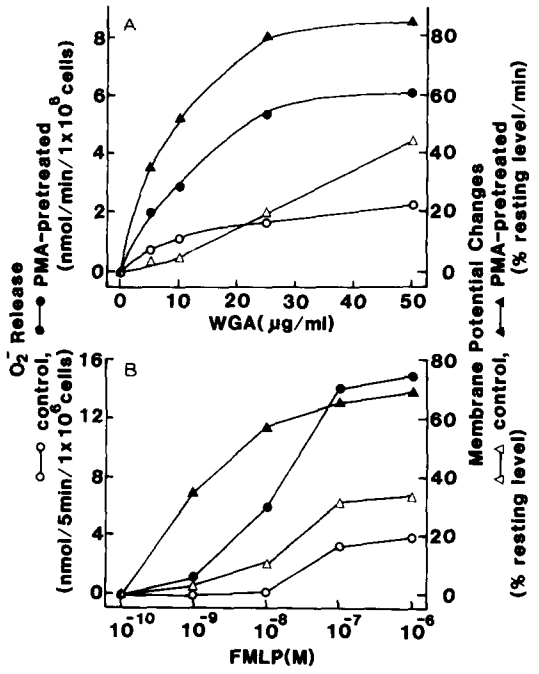


Fig. 5. Effect of phorbol myristate acetate on $O_2^-$ release and membrane potential changes stimulated by wheat-germ agglutinin or fMet-Leu-Phe. The cells ($1 \cdot 10^6$/ml) were preincubated with or without phorbol myristate acetate (0.25 ng/ml) for 5 min at 37 °C, and stimulated by the indicated concentrations of wheat germ agglutinin (A) of fMet-Leu-Phe (B). The values of $O_2^-$ release and membrane potential changes stimulated by phorbol myristate acetate alone were subtracted from those stimulated by wheat-germ agglutinin or fMet-Leu-Phe (see Figs. 2 and 4). Data are expressed as means of 3–5 experiments, each done in triplicate. FMLP, fMet-Leu-Phe; PMA, phorbol myristate acetate; WGA, wheat-germ agglutinin.

dose-response curves for triggering of $O_2^-$ release and membrane depolarization by wheat-germ agglutinin in phorbol myristate acetate-pretreated or control cells were identical.

The similar findings were also observed when fMet-Leu-Phe, a chemotactic peptide, was used as stimulus. As shown in Fig. 4B, phorbol myristate acetate potentiated the initial rate as well as the total amount of $O_2^-$ release, and the initial rate as well as the maximal change in membrane potential stimulated by fMet-Leu-Phe. No demonstrable lag time was detected in fMet-Leu-Phe-induced $O_2^-$ release and membrane depolarization. The enhancement of $O_2^-$ release and membrane depolarization by phorbol myristate acetate was

observed over the complete range of effective f-Met-Leu-Phe concentrations ($10^{-9}$–$10^{-6}$ M). In addition, the dose-response curves for triggering of $O_2^-$ release and membrane depolarization by fMet-Leu-Phe in phorbol myristate acetate-pretreated or control cells were identical (Fig. 5B).

*Effect of phorbol myristate acetate on stimulus-induced increase in $[Ca^{2+}]_i$*

As shown in Fig. 6, Con A induced an increase in $[Ca^{2+}]_i$ with a lag of $23 \pm 5$ s ($n = 6$), whereas fMet-Leu-Phe induced an increase in $[Ca^{2+}]_i$ without a demonstrable lag. Thus, when Con A was used as stimulus, an increase in $[Ca^{2+}]_i$ apparently preceded the onset of membrane depolarization and $O_2^-$ release. On the other hand, when fMet-Leu-Phe was used as stimulus, it was difficult to assess the sequence of these responses. Wheat-germ agglutinin also induced a rapid increase in $[Ca^{2+}]_i$ without a demonstrable lag (data not shown).

To test the possibility that a low concentration of phorbol myristate acetate enhances $O_2^-$ release and membrane depolarization by augmenting an increase in $[Ca^{2+}]_i$, we studied the effect of phorbol myristate acetate on the change in $[Ca^{2+}]_i$ stimulated by Con A or fMet-Leu-Phe. The change in $[Ca^{2+}]_i$ stimulated by Con A or fMet-Leu-Phe was calculated by subtracting the values of $[Ca^{2+}]_i$ immediately before the addition of each stimulus from the maximal levels of $[Ca^{2+}]_i$ induced by the stimulus. As shown in Figs. 6 and 7, the pretreatment of cells with 0.25 ng/ml phorbol myristate acetate slightly enhanced the increase in $[Ca^{2+}]_i$ stimulated by Con A ($138 \pm 24\%$ of control; $P < 0.01$, $n = 4$), but did not affect the increase in $[Ca^{2+}]_i$ stimulated by fMet-Leu-Phe ($92 \pm 22\%$ of control; $n = 3$). The level of $[Ca^{2+}]_i$ was slightly decreased by the addition of phorbol myristate acetate. The decreased level of $[Ca^{2+}]_i$ before the addition of Con A might be responsible for the apparent enhancement of Con A-induced increase in $[Ca^{2+}]_i$ by the low concentration of phorbol myristate acetate. Then, the change in $[Ca^{2+}]_i$ stimulated by Con A was calculated by subtracting the values of $[Ca^{2+}]_i$ immediately before the addition of phorbol myristate acetate from the maximal levels of $[Ca^{2+}]_i$ induced by Con A. The results showed that the low concentration of

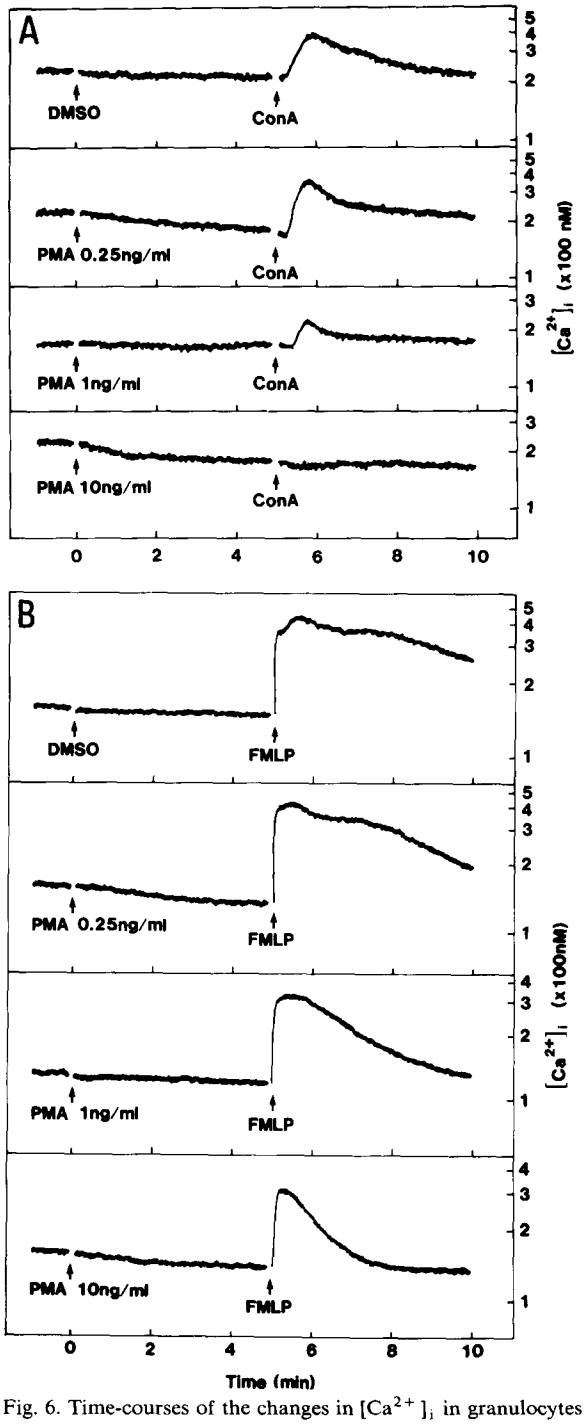


Fig. 6. Time-courses of the changes in $[Ca^{2+}]_i$ in granulocytes stimulated by the sequential addition of phorbol myristate acetate and Con A (A), or fMet-Leu-Phe (B). Phorbol myristate acetate (0.25–10 ng/ml) was added initially and 5 min thereafter Con A (100 μg/ml) or fMet-Leu-Phe ($10^{-7}$ M) was added. The same concentration of DMSO was added to the control cells. Final cell concentration was $5 \cdot 10^6$ cells/ml. FMLP, fMet-Leu-Phe; PMA, phorbol myristate acetate.

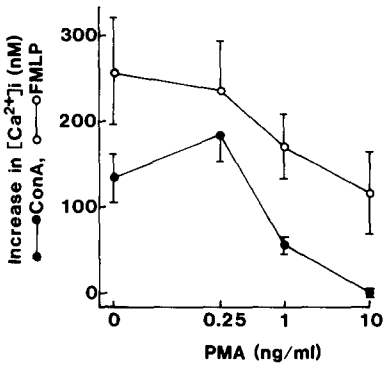


Fig. 7. Effect of phorbol myristate acetate on the increase in $[Ca^{2+}]_i$ stimulated by Con A or fMet-Leu-Phe. The cells ($5 \cdot 10^6$/ml) were preincubated with or without indicated concentrations of phorbol myristate acetate (0.25, 1 or 10 ng/ml) for 5 min at 37 °C, and stimulated by Con A (100 μg/ml) or fMet-Leu-Phe ($10^{-7}$ M). The increase in $[Ca^{2+}]_i$ was calculated by subtracting the values of $[Ca^{2+}]_i$ immediately before the addition of stimuli from the maximal levels of $[Ca^{2+}]_i$ induced by the stimuli (see Fig. 6). Data from three or four experiments are shown, and expressed as the increase in $[Ca^{2+}]_i$. Each experiment was done in duplicate or triplicate. FMLP, fMet-Leu-Phe; PMA, phorbol myristate acetate.

phorbol myristate acetate (0.25 ng/ml) significantly enhanced the increase in $[Ca^{2+}]_i$ stimulated by Con A (133 ± 30% of control; $P < 0.05$). The lag time between addition of Con A and onset of an increase in $[Ca^{2+}]_i$ in phorbol myristate acetate-pretreated cells was 24 ± 3 s ($n = 6$), and was not reduced as compared with that in control cells (23 ± 5 s; $n = 6$). Moderate or high concentrations of phorbol myristate acetate (above 1 ng/ml) inhibited an increase in $[Ca^{2+}]_i$ stimulated by Con A or fMet-Leu-Phe in a dose-dependent manner (Figs. 6 and 7). The fMet-Leu-Phe-induced increase in $[Ca^{2+}]_i$ was partially inhibited by pretreatment of cells with 10 ng/ml phorbol myristate acetate, whereas the Con A-induced increase in $[Ca^{2+}]_i$ was completely abolished. Phorbol myristate acetate by itself did not induce an increase in $[Ca^{2+}]_i$ at the concentrations used in the present experiments (up to 10 ng/ml).

*Effect of phorbol myristate acetate on $O_2^-$ release, membrane potential changes and an increase in $[Ca^{2+}]_i$ stimulated by the calcium ionophore, ionomycin*

$O_2^-$ release and membrane depolarization stimulated by the receptor-mediated agonists (Con A, wheat-germ agglutinin and fMet-Leu-Phe) were markedly enhanced by the pretreatment of cells with a low concentration of phorbol myristate acetate, whereas an increase in $[Ca^{2+}]_i$ stimulated by these agonists was not affected or slightly enhanced. These findings suggest that phorbol myristate acetate affects primarily the pathway distal to the increase in $[Ca^{2+}]_i$ or the pathway independent of the $Ca^{2+}$-mobilizing system. To obtain additional evidence, we studied the effect of phorbol myristate acetate on $O_2^-$ release, membrane potential changes and an increase in $[Ca^{2+}]_i$ stimulated by the $Ca^{2+}$ ionophore, ionomycin, which bypasses the receptors and increases directly the level of $[Ca^{2+}]_i$. The representative time-courses of $O_2^-$ release, membrane potential changes and an increase in $[Ca^{2+}]_i$ stimulated by the sequential addition of phorbol myristate acetate and ionomycin are shown in Fig. 8. Ionomycin (1 μM) induced a rapid increase in $[Ca^{2+}]_i$ without a demonstrable lag, which was followed by membrane depolarization with a lag of 15 ± 3 s ($n = 7$), and by $O_2^-$ release with a lag of 22 ± 5 s ($n = 3$). Thus, the sequence of responses stimulated by ionomycin was an increase in $[Ca^{2+}]_i$, membrane depolarization and $O_2^-$ release. The pretreatment of cells with 0.25 ng/ml phorbol myristate acetate enhanced not only $O_2^-$ release but also membrane depolarization stimulated by ionomycin, but did not affect the increase in $[Ca^{2+}]_i$. The lag time between addition of ionomycin and onset of membrane depolarization in phorbol myristate acetate-pretreated cells was 12 ± 1 s ($n = 3$), and was significantly ($P < 0.02$) reduced as compared with that in control cells. The lag time between addition of ionomycin and onset of $O_2^-$ release in phorbol myristate acetate-pretreated cells was 16 ± 2 s ($n = 3$), and significantly ($P < 0.01$) reduced as compared with that in control cells. A high concentration of phorbol myristate acetate (10 ng/ml) did not impair the increase in $[Ca^{2+}]_i$ stimulated by ionomycin.

## Discussion

Exposure of granulocytes to a variety of soluble stimuli results in early changes in $[Ca^{2+}]_i$ and in transmembrane potential [10–16]. Both changes are proposed to play an important role in activa-

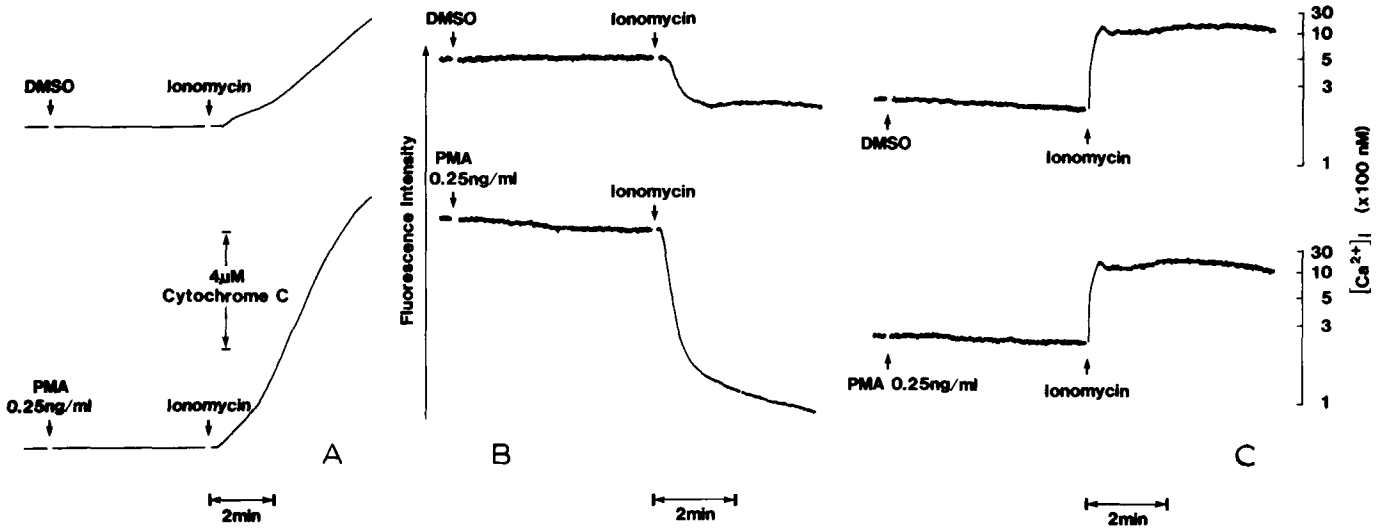


Fig. 8. Time-courses of $O_2^-$ release (A), membrane potential changes (B) and the changes in $[Ca^{2+}]_i$ (C) in granulocytes stimulated by the sequential addition of phorbol myristate acetate and ionomycin. Phorbol myristate acetate (0.25 ng/ml) was added initially and 5 min thereafter ionomycin (1 $\mu$M) was added. The same concentration of DMSO was added to the control cells. Final cell concentration was $1 \cdot 10^6$ cells/ml for measurement of $O_2^-$ release and membrane potential changes, and $5 \cdot 10^6$ cells/ml for measurement of the changes in $[Ca^{2+}]_i$, respectively, PMA, phorbol myristate acetate.

tion of cell metabolism. However, the causal relationship between these two ionic changes is still unclear and the molecular mechanism underlying membrane depolarization remains unresolved. When Con A or ionomycin was used as stimulus, an increase in $[Ca^{2+}]_i$ clearly preceded the onset of membrane depolarization. This finding raises the possibility that an increase in $[Ca^{2+}]_i$ may trigger membrane depolarization. By using the patch clamp method, Von Tscharner et al. [20] have recently reported that cation channels in human granulocytes are activated by an increase in $[Ca^{2+}]_i$, although these channels are non-selective and permeable to $Na^+$, $K^+$ and $Ca^{2+}$. Thus, it is possible that influx of $Na^+$ through these channels may, at least in part, account for membrane depolarization stimulated by an increase in $[Ca^{2+}]_i$. However, it is unlikely that membrane depolarization is directly stimulated by an increase in $[Ca^{2+}]_i$, since there was no direct correlation between the magnitude of an increase in $[Ca^{2+}]_i$ and membrane depolarization, and both responses were differently modulated by a low concentration of phorbol myristate acetate. Membrane depolarization stimulated by various agonists was markedly potentiated by pretreatment of cells with a low concentration of phorbol myristate acetate, whereas an increase in $[Ca^{2+}]_i$ was not affected or minimally potentiated. Furthermore, the lag time between addition of the stimulus (Con A or ionomycin) and the onset of membrane depolarization was significantly reduced by pretreatment of cells with a low concentration of phorbol myristate acetate, whereas the lag time between addition of Con A and onset of the increase in $[Ca^{2+}]_i$ was not affected. These findings indicate that a low concentration of phorbol myristate acetate may potentiate membrane depolarization by acting primarily at the post-receptor level, in particular, at the level distal to an increase in $[Ca^{2+}]_i$, but not by augmenting an increase in $[Ca^{2+}]_i$. The differential effect on membrane depolarization and an increase in $[Ca^{2+}]_i$ has been also reported by using cyclic AMP. Cyclic AMP inhibits membrane depolarization without affecting an increase in $[Ca^{2+}]_i$ in human granulocytes stimulated by fMet-Leu-Phe [21]. These findings taken together suggest that membrane depolarization could be indirectly stimulated by an increase in $[Ca^{2+}]_i$ through a certain process which is susceptible to cyclic AMP and a low concentration of phorbol myristate acetate. The possible candidates for this process may include activation of calmodulin-dependent protein kinases or phospholipase C [22,23]. The reduced lag time between addition of the stimulus (Con A or ionomycin) and onset of membrane depolarization implies that the metabolism triggered by an increase in $[Ca^{2+}]_i$ may be accelerated by a low concentration of phorbol myristate acetate. Moderate or high concentrations of phorbol myristate acetate stimulated membrane depolarization without inducing an increase in $[Ca^{2+}]_i$, suggesting that membrane depolarization could be also stimulated by activation of protein kinase C. It remains to be determined whether the same provoking system of membrane depolarization is activated by an increase in $[Ca^{2+}]_i$ and protein kinase C. We have previously reported that human granulocytes gain the ability to exhibit membrane depolarization in response to the $Ca^{2+}$ ionophore A23187 and phorbol myristate acetate as the cells differentiate and that membrane depolarization induced by either A23187 or phorbol myristate acetate is inhibited by 2-deoxyglucose [13]. In this regard, membrane depolarization induced by the $Ca^{2+}$ ionophore is similar to that induced by phorbol myristate acetate.

Ionomycin induced a rapid increase in $[Ca^{2+}]_i$ without a demonstrable lag, which was followed by membrane depolarization and $O_2^-$ release with a distinct lag time. Both fMet-Leu-Phe and wheat-germ agglutinin, like ionomycin, induced a rapid increase in $[Ca^{2+}]_i$ without a demonstrable lag. However, in contrast to ionomycin, no demonstrable lag was seen in fMet-Leu-Phe- or wheat germ agglutinin-induced membrane depolarization and $O_2^-$ release. These findings suggest that, at least, the initial rapid onset of membrane depolarization and $O_2^-$ release induced by fMet-Leu-Phe or wheat germ agglutinin are neither directly stimulated by an increase in $[Ca^{2+}]_i$ nor stimulated by the synergistic action of protein kinase C and an increase in $[Ca^{2+}]_i$ [24], and should be explained by other mechanisms activated by receptor stimulation. The present results suggest that these mechanisms are also susceptible to

a low concentration of phorbol myristate acetate, since phorbol myristate acetate markedly potentiated the initial rapid onset of membrane depolarization and $O_2^-$ release stimulated by fMet-Leu-Phe or wheat-germ agglutinin. The similar findings are also reported in exocytosis from human granulocytes. fMet-Leu-Phe-induced exocytosis starts without a demonstrable lag, whereas ionomycin-induced exocytosis starts with a distinct lag time [25].

In contrast to an increase in $[Ca^{2+}]_i$, $O_2^-$ release and membrane depolarization stimulated by various agonists were markedly potentiated in parallel by pretreatment of cells with a low concentration of phorbol myristate acetate. The dose-response curves for triggering of $O_2^-$ release and membrane depolarization by Con A, wheat-germ agglutinin or fMet-Leu-Phe in phorbol myristate acetate-pretreated or control cells were identical. Furthermore, the lag time between addition of the stimulus (Con A or ionomycin) and the onset of membrane depolarization or $O_2^-$ release was significantly reduced by pretreatment of cells with a low concentration of phorbol myristate acetate. These findings suggest that the system provoking membrane depolarization and the system activating NADPH oxidase share a common pathway, and that both systems are stimulated and regulated in a similar manner. These findings are consonant with our previous observations that the $O_2^-$-producing system and the system provoking membrane potential changes develop concomitantly as human granulocytes mature and differentiate [13], and that both systems develop or decline concomitantly during activation or deactivation of mouse peritoneal macrophages [18].

Granulocytes could be primed in vivo by chemotactic factors during migration to the inflammatory sites. The chemotactic peptide, fMet-Leu-Phe, stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form inositol triphosphate and diacylglycerol, which can activate protein kinase C [24]. However, recent evidence suggests that fMet-Leu-Phe-induced activation of the respiratory burst is independent of activation of protein kinase C [26–29], although diacylglycerol is accumulated after stimulation with fMet-Leu-Phe [30]. These findings and the present experiments taken together suggest that diacylglycerol accumulated in the cell might work as a priming agent in the physiological states, even when the concentration of diacylglycerol by itself is too low to activate the respiratory burst.

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