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Tyrosine phosphorylation is involved in the respiratory burst of electropermeabilized human neutrophils at a step before diacylglycerol formation by phospholipase C

Takashi Mitsuyama, Koichiro Takeshige and Shigeki Minakami

Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812, Japan

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We studied a step where tyrosine phosphorylation is involved in a signaling pathway for the activation of the superoxide (O_2^-)-generating NADPH oxidase using electropermeabilized human neutrophils. The permeabilized cells produced O_2^- by the addition of a protein tyrosine phosphatase inhibitor, vanadate, as well as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and protein kinase C (PKC) activators such as phorbol myristate acetate (PMA) and L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol (OAG). The O_2^- production by the stimulants was completely inhibited by PKC inhibitors such as calphostin C and staurosporine and was not affected by 1% ethanol, a metabolic modulator of phospholipase D (PLD). Furthermore, the O_2^- production by vanadate and fMLP, but not by OAG and PMA, was inhibited by both an inhibitor of phospholipase C (PLC), neomycin, and an inhibitor of tyrosine kinase, ST-638. These findings suggest that tyrosine phosphorylation is involved in the activation of the oxidase at a step before diacylglycerol formation by PLC, and that PLD may not be involved in the signaling pathway in permeabilized cells.

Human neutrophil; Respiratory burst; Electropermeabilization; Signal transduction; Tyrosine phosphorylation; Phospholipase C

1. INTRODUCTION

Neutrophils are important in the host defense against microbial infection [1] and for this purpose, are equipped with an enzymatic complex, NADPH oxidase, which is able to catalyze the one-electron reduction of molecular oxygen to superoxide (O_2^-). The oxidase is dormant in non-activated neutrophils and the signal transduction process leading to the activation of the oxidase has been extensively studied [2,3]. It is generally accepted that binding of agonists to their receptors stimulates phospholipase C (PLC) through the activation of a GTP-binding protein. The PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into the calcium ion mobilizer, inositol 1,4,5-trisphosphate, and the protein kinase C (PKC) activator, 1,2-diacylglycerol (DAG). The activation of PKC leads to the conversion of dormant oxidase into its active form in an unknown manner. Recently, it has been reported that tyrosine phosphorylation is involved in the signaling pathway [4–13]. Grinstein et al. [11] and Trudel et al. [12,13] have reported that neutrophils and granulocytic HL60 cells possess constitutively active tyrosine kinases, and phosphoprotein accumulation is normally prevented by vigorous concomitant protein tyrosine phosphatase activity, and that vanadate induces the accumulation of tyrosine-phosphorylated proteins by inhibiting the protein tyrosine phosphatase activity with close correlation to the induction of the respiratory burst. *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP) also induces tyrosine phosphorylation on several proteins, and the incubation of neutrophils with tyrosine kinase inhibitors, such as ST-638 and erbstatin, decreases both the amount of tyrosine phosphorylation and O_2^- production induced by fMLP [5–9]. The exact step affected by tyrosine phosphorylation in the signaling pathway, however, has not been identified.

Permeabilized cells are useful for investigating the signaling pathway because they have pores on the plasma membrane through which agents with molecular weights below 1,000 Da permeate, and which produce O_2^- on stimulation by vanadate, fMLP and PKC activators [11–16]. We have recently reported that cyclic AMP inhibits the respiratory burst at a site downstream of PKC [17]. The present studies were undertaken to clarify the step of tyrosine phosphorylation in the signaling pathway for the respiratory burst. For this purpose, we examined the effects of inhibitors of PKC, PLC and tyrosine kinase on O_2^- production induced by vanadate, fMLP, and PKC activators such as phorbol myristate acetate (PMA) and L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol (OAG), using electropermeabilized human

Correspondence address: T. Mitsuyama, Department of Biochemistry, Kyushu University School of Medicine, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan. Fax: (81) (92) 632-2373.

Abbreviations: DAG, 1,2-diacylglycerol; PIP_2, L-α-phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; MAP kinase, mitogen-activated protein kinase.

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neutrophils. We also studied the involvement of phospholipase D (PLD) in the signaling pathway of permeabilized cells using ethanol, a metabolic modulator of PLD, because PLD has been reported to be involved in the pathway [18–24]. The results suggest that tyrosine phosphorylation occurs at a step before DAG formation by PLC, and that PLD is not involved in the pathway for activating the respiratory burst of permeabilized cells.

2. MATERIALS AND METHODS

2.1. *Reagents*

Staurosporine and calphostin C were purchased from Kyowa Medex, Tokyo, Japan. NADPH and ATP were from Oriental Yeast, Tokyo, Japan. OAG was from Funakoshi Chemical, Tokyo, Japan. SDS was from Wako Pure Chemical Industries, Osaka, Japan. The following materials were obtained from Sigma Chemical Co., St. Louis, MO, USA: L-α-phosphatidylinositol 4,5-bisphosphate (PIP_2), ferricytochrome *c*, superoxide dismutase (SOD), PMA, fMLP, neomycin sulfate and sodium orthovanadate. α-Cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST-638) was generously given by Kanegafuchi Chemical Industry, Tokyo, Japan. All other reagents were of analytical grade.

2.2. *Preparation of human neutrophils*

Human neutrophils were isolated from a healthy donor as described previously [25]. In short, after elimination of erythrocytes by dextran sedimentation followed by a brief hypotonic lysis, the cell suspension was centrifuged in a Ficoll–sodium iothalamate gradient to separate the polymorphonuclear leukocytes from lymphocytes, monocytes and platelets. Isolated cells were suspended in a HEPES-buffered salt solution containing 135 mM NaCl, 5 mM KCl, 5 mM glucose and 20 mM HEPES (pH 7.4) and stored on ice until use. For electropermeabilization, cells were suspended in an ice-cold permeabilization medium containing 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.193 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 6.7).

2.3. *Cell permeabilization*

Permeabilization was performed according to the method of Lu and Grinstein [16] except that the ice-cold permeabilization medium was adjusted to pH 6.7 (4°C). Briefly, 8×10^6 cells were transfered to a Bio-Rad Gene Pulser and permeabilized with two discharges of 5 kV/cm from a 25-microfarad capacitor. Between pulses, cells were rapidly sedimented and resuspended in the fresh ice-cold medium. Permeabilized cells were sedimented and finally suspended in an assay buffer containing 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.193 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 6.7 at 37°C). Cells (2×10^6) were used for each assay within 15 min of preparation.

2.4. *Acetylation of ferricytochrome* c

Ferricytochrome *c* was acetylated as previously described [26]. At 4°C, 200 mg of ferricytochrome *c* was dissolved in 10 ml of a half-saturated solution of sodium acetate. After 0.4 ml of acetic anhydride was added and stirred for 30 min, the reaction mixture was dialyzed at 4°C for 12 h against the assay buffer.

2.5. *Assay of the O_2^- production by intact neutrophils*

The assay mixture (1.0 ml) consisted of 50 μM ferricytochrome *c*, 1 mM $CaCl_2$ and 1×10^6 cells in the HEPES-buffered salt solution (see section 2.2). Cells were incubated at 37°C for 5 min and O_2^- production was initiated by the addition of a stimulant and measured by determining the rate of superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction at 550–540 nm using a dual-wavelength spectrophotometer (Hitachi 556) as previously described [25]. The O_2^- release was calculated using a molar absorption coefficient of 19,100 $M^{-1} \cdot cm^{-1}$.

2.6. *Assay of the O_2^- production by electropermeabilized neutrophils*

The assay mixture (1.0 ml) consisted of 50 μM acetylated cytochrome *c* and 2×10^6 cells in the assay buffer (see section 2.3). Under this condition, the Ca^{2+} concentration in the reaction mixture was about 100 nM [15]. Cells were incubated at 37°C for 5 min followed by the addition of 2 mM NADPH and 1 mM ATP. The production of O_2^- was initiated by the addition of a stimulant and measured by determining the rate of SOD-inhibitable reduction of acetylated cytochrome *c* at 550–540 nm using a dual-wavelength spectrophotometer (Hitachi 556). The O_2^- release was calculated using a molar absorption coefficient of 19,100 $M^{-1} \cdot cm^{-1}$.

3. RESULTS

3.1. *Effects of PKC inhibitors on the O_2^- production*

To investigate the step of tyrosine phosphorylation in the signaling pathway for activating NADPH oxidase, we examined the effects of PKC inhibitors on the O_2^- production of electropermeabilized human neutrophils induced by a protein tyrosine phosphatase inhibitor, vanadate, and other agents such as fMLP, OAG and PMA. The O_2^- production by the stimulants was dose-dependently inhibited by PKC inhibitors such as calphostin C and staurosporine, and the production was completely abolished by 5 μM calphostin C (Table I) and 10 nM staurosporine (results not shown). In the present study, we used calphostin C and staurosporine as PKC inhibitors because it had been reported that they inhibit PKC by different mechanisms and that calphostin C is a relatively specific inhibitor for PKC, although staurosporine may also inhibit other protein kinases [27–29]. The NADPH oxidase activity itself was not affected by the inhibitors because the O_2^- production of permeabilized cells by SDS was inhibited by neither 5 μM calphostin C nor 10 nM staurosporine [17]. These results suggest that the signal transduction process lead-

Table I

Effects of ethanol and calphostin C on O_2^- production of permeabilized neutrophils

Stimulants	Pretreatments		
	Calphostin C (2 μM) (% of control)	Calphostin C (5 μM) (% of control)	Ethanol (1%) (% of control)
fMLP	9.3 ± 6.6	0.0 ± 0.0	94.1 ± 4.3
Vanadate	16.7 ± 6.2	0.0 ± 0.0	91.4 ± 0.7
OAG	42.3 ± 5.0	0.0 ± 0.0	95.0 ± 4.3
PMA	37.3 ± 7.6	0.0 ± 0.0	94.6 ± 4.0

Permeabilized neutrophils (2×10^6/ml) were preincubated with ethanol and calphostin C for 1 min and 5 min, respectively, at 37°C and then stimulated by 1 μM fMLP, 10 μM vanadate, 10 μM OAG or 10 ng/ml PMA. The O_2^- production was measured as described in section 2. The activities are given as the percentages of control activities. The control activities of permeabilized cells by fMLP, vanadate, OAG and PMA were 0.93 ± 0.08, 1.39 ± 0.06, 2.55 ± 0.05 and 2.80 ± 0.19 nmol/min/10^6 cells, respectively. The data represent the means ± S.D. of three separate experiments.

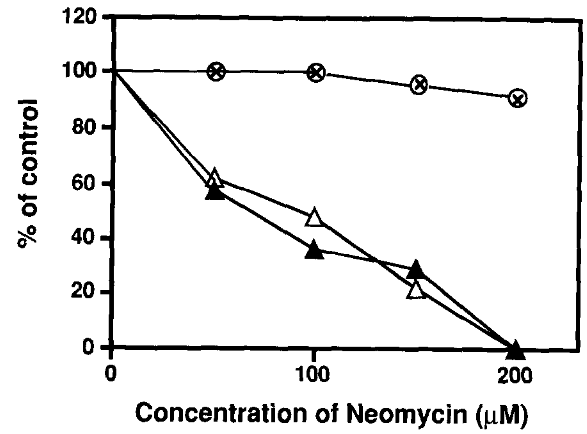

Fig. 1. Effect of neomycin on O_2^- production by various stimulants in permeabilized neutrophils. The O_2^- production was measured as described in section 2 except that neomycin at various concentrations was added to the assay mixture 1 min before the addition of a stimulant. The results are representative of at least three independent experiments. The activities are given as the percentages of control activities. The control activities of the O_2^- production by 1 μM fMLP (△), 10 μM OAG (×), 10 ng/ml PMA (○) and 10 μM vanadate (▲) were 0.76, 1.70, 1.70 and 1.78 nmol/min/10^6 cells, respectively.

ing to the respiratory burst induced by vanadate, fMLP, OAG and PMA is completely dependent on PKC, and that the step of tyrosine phosphorylation responsible for the O_2^- production induced by vanadate is located upstream of PKC. As shown in Table I, 2 μM of calphostin C more strongly inhibited the fMLP- and vanadate-induced O_2^- production than the PMA- and OAG-induced production. This may be due to the fact that calphostin C acts on the regulatory domain of PKC and the concentration of PMA or OAG might be higher than that of the diacylglycerol produced on stimulation by fMLP or vanadate, whereas staurosporine inhibited the O_2^- production induced by fMLP, vanadate, PMA

Table II

Effects of phosphatidylinositol 4,5-bisphosphate on the neomycin-inhibited O_2^- production of permeabilized neutrophils

Condition	Stimulants (% of control)	
	fMLP	Vanadate
+ neomycin	24.0 ± 3.6	24.0 ± 3.7
+ 10 μg PIP_2	105.3 ± 2.1	93.0 ± 5.0
+ neomycin + 5 μg PIP_2	51.3 ± 9.0	47.3 ± 6.3
+ neomycin + 10 μg PIP_2	85.7 ± 5.4	74.0 ± 2.9

The O_2^- production was measured as described in section 2. The permeabilized neutrophils (2×10^6/ml) were preincubated with either 150 μM neomycin for 1 min or phosphatidylinositol 4,5-bisphosphate (PIP_2) for 5 min or both at 37°C and then stimulated by 1 μM fMLP or 10 μM vanadate. The activities are given as the percentages of control activities. The control activities of permeabilized cells by fMLP and vanadate were 1.05 ± 0.14 and 1.63 ± 0.35 nmol/min/10^6 cells, respectively. The data represent the means ± S.D. of three separate experiments.

and OAG in similar dose-dependent manners, probably by its action on the catalytic domain of PKC (results not shown).

3.2. *Effects of neomycin and ethanol on the O_2^- production*

The effect of an inhibitor of PLC, neomycin, on the O_2^- production by permeabilized cells was examined to further investigate the step where tyrosine phosphorylation responsible for O_2^- production induced by vanadate occurs in the signaling pathway. As shown in Fig. 1, neomycin inhibited the production by vanadate and fMLP in a similar dose-dependent manner, and 200 μM neomycin completely inhibited the production but only slightly inhibited the production of OAG and PMA. These results suggest that the step of tyrosine phosphorylation is upstream of the DAG formation by PLC. Although neomycin inhibits PLC by binding its substrates, inositol phospholipids, and has been used to show the involvement of PLC in various cellular responses [30,31], it also affects other components of signal transduction systems at high concentrations [32–34]. In the present study, however, the inhibition by neomycin seems to be due to the inhibition of phosphoinositide breakdown by PLC because the concentration of neomycin used was low enough and the inhibition by neomycin was remarkably restored by the addition of 10 μg PIP_2 (Table II).

We also investigated whether phospholipase D (PLD) is involved in the signaling pathway for the respiratory burst of permeabilized cells because DAG may be generated not only by PLC but by PLD. In the presence of ethanol, the activation of PLD results in the formation of phosphatidylethanol through a transphosphatidylation reaction, together with a decrease in phosphatidic acid (PA) generation. It has been reported that 1% ethanol severely inhibits the O_2^- production mediated by PLD but not by phosphatidylinositol-specific PLC (PI-PLC) [21]. As shown in Table I, 1% ethanol hardly inhibited the O_2^- production of permeabilized cells by vanadate, fMLP, OAG and PMA, indicating that PLD seems not to be involved in the respiratory burst of the permeabilized cells under our experimental conditions.

3.3. *Effect of ST-638 on the O_2^- production*

Tyrosine phosphorylation has been supposed to be essential for the activation of the oxidase induced by fMLP in intact cells [5–9], which is partially based on the studies using tyrosine kinase inhibitors such as ST-638 and erbstatin. We ascertained whether tyrosine phosphorylation might be essential for O_2^- production of permeabilized cells as well as fMLP-induced production in intact cells. As shown in Fig. 2, ST-638 dose-dependently inhibited O_2^- production not only by vanadate but by fMLP, and 15 μM ST-638 completely inhibited the production, whereas 15 μM ST-638 inhibited the production by OAG and PMA by only about 20%

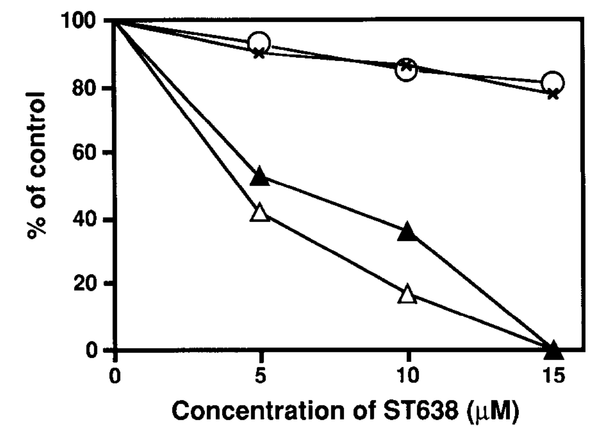

Fig. 2. Effect of ST-638 on O_2^- production by various stimulants in permeabilized neutrophils. The O_2^- production was measured as described in Fig. 1 except that ST-638 at various concentrations was added to the assay mixture 1 min before the addition of a stimulant. The results are representative of at least three independent experiments. The activities are given as the percentages of control activities. The control activities by fMLP (△), OAG (×), PMA (○) and vanadate (▲) were 0.72, 2.22, 2.22 and 2.01 nmol/min/10^6 cells, respectively.

(Fig. 2), indicating that tyrosine phosphorylation induced by fMLP, as well as by vanadate, was essential and occurs a step before DAG formation. These results support our hypothesis that tyrosine phosphorylation is involved in the signaling pathway for the respiratory burst at a step before DAG formation by PLC.

4. DISCUSSION

In the present study, we focused on a step where tyrosine phosphorylation is involved in the signaling pathway for the activation of the NADPH oxidase using electropermeabilized human neutrophils. The results suggest that tyrosine phosphorylation is necessary for O_2^- production by fMLP but not by PMA or OAG, indicating that tyrosine phosphorylation is required to activate PKC upon the receptor-dependent stimulation. This is based on the following observations: (i) the O_2^- production by fMLP and vanadate, but not by PMA and OAG, was inhibited by an inhibitor of tyrosine kinase, ST-638 (Fig. 2), (ii) the O_2^- production by fMLP, vanadate, PMA and OAG was inhibited by PKC inhibitors such as calphostin C (Table I) and staurosporine (results not shown). Furthermore, the present study suggests that tyrosine phosphorylation is required at a step before DAG formation by PLC because the O_2^- production by fMLP and vanadate, but not by PMA and OAG, was inhibited by an inhibitor of PLC, neomycin (Fig. 1). The inhibition by neomycin seems to be due to the inhibition of phosphoinositide breakdown by PLC, because the inhibition by neomycin was remarkably restored by the addition of PIP_2 (Table II). The enzymes or proteins affected by the tyrosine phosphorylation, however, could not be identified. A possible enzyme may be PLC because tyrosine phosphorylation of PI-PLCγ enhances its activity [35,36] and mRNA for PLCγ_2 is expressed in HL-60 cells [37,38], although several types of PLC exist in human neutrophils [3]. $Gi_{\alpha 2}$, which is involved in PLC activation and is tyrosine-phosphorylated in activated neutrophils [5], may also be a candidate. Although it has been proposed that tyrosine phosphorylation induces the activation of PLD [4] or of mitogen-activated protein kinase (MAP kinase) [10], PLD was not involved in the signaling pathway under our experimental conditions, and MAP kinase has been supposed to be located at the downstream of PLC [10]. The involvement of tyrosine phosphorylation on phosphatidylinositol 3-kinase (PI 3-kinase) is unlikely because PI 3-kinase in neutrophils is stimulated by fMLP without tyrosine phosphorylation [39].

The signaling pathway of neutrophils might be modified by the procedure of the electropermeabilization because of the following reasons. First, staurosporine inhibited the O_2^- production of intact neutrophils stimulated by fMLP far less than that by OAG and PMA (results not shown), indicating that the production by fMLP was partially mediated by the PKC-independent pathway in accordance with previous reports [15,16,40], whereas the production by permeabilized cells stimulated by fMLP, OAG and PMA was inhibited by staurosporine in a similar dose-dependent manner. Thus, the PKC-independent pathway seems to be activated by fMLP in intact cells but not in permeabilized cells. Second, 1% ethanol caused about 80% inhibition of the fMLP-induced O_2^- production with or without cytochalasin B, and about 40% inhibition of the OAG- or PMA-induced O_2^- production in intact cells (results not shown). These results are compatible with a hypothesis that fMLP and PMA activate PLD [18–23], and that the PLD-mediated breakdown of phospholipids is induced by fMLP even in the absence of cytochalasin B [24]. Table I, however, showed that the production by the permeabilized cells stimulated by fMLP, OAG or PMA was not affected by 1% ethanol. The discrepancy of the effects of ethanol between the intact and permeabilized cells may be due to differences in the intracellular free Ca^{2+} concentrations. The concentration in permeabilized cells was essentially fixed to the physiological concentration at a resting stage of neutrophils, 100 nM, and the activation of PLD is strictly Ca^{2+}-dependent and requires the increase in the intracellular free Ca^{2+} concentration to about 1 μM [23].

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