Korean J. of Pharmacology
Vol. 32, No. 1, pp. 103~112, 1996

Alteration of the Activated Responses in Platelet-Activating Factor-Stimulated Neutrophils by Protein Kinase Inhibitors

Kang-Kun Lee, Ji-Young Ko, Dong-Suk Ham
Yong-Kyoo Shin and Chung-Soo Lee*

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

ABSTRACT

Roles of protein kinase C and protein tyrosine kinase in the activation of neutrophil respiratory burst, degranulation and elevation of cytosolic Ca^{2+} in platelet-activating factor (PAF)-stimulated neutrophils were investigated.

Superoxide and H_2O_2 production and myeloperoxidase and acid phosphatase release in PAF-stimulated neutrophils were inhibited by protein kinase C inhibitors, staurosporine and H-7 and protein tyrosine kinase inhibitors, genistein and tyrphostin. The PAF-induced elevation of $[Ca^{2+}]_i$ in neutrophils was inhibited by staurosporine, genistein and methyl-2,5-dihydroxycinnamate. Staurosporine inhibited both intracellular Ca^{2+} release and Mn^{2+} influx in PAF-stimulated neutrophils. Genistein and methyl-2,5-dihydroxycinnamate inhibited Mn^{2+} influx induced by PAF, whereas their effects on intracellular Ca^{2+} release were not detected. In neutrophils preactivated by PMA, the stimulatory effect of PAF on the elevation of $[Ca^{2+}]_i$ was reduced.

Protein kinase C and protein tyrosine kinase may be involved in respiratory burst, lysosomal enzyme release and Ca^{2+} mobilization in PAF-stimulated neutrophils. The elevation of $[Ca^{2+}]_i$ appears to be accomplished by intracellular Ca^{2+} release and Ca^{2+} influx which are differently regulated by protein kinases. Preactivation of protein kinase C appears to attenuate the stimulatory action of PAF on intracellular Ca^{2+} mobilization.

Key Words: Protein kinase inhibitor, Platelet-activating factor, Neutrophil responses

INTRODUCTION

Platelet-activating factor (PAF) is 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkyl acetyl GPC). PAF is synthesized and released by platelets, neutrophils, macrophages, mast cells, basophils, eosinophils and endothelial cells (Braquet *et al.*, 1987). PAF stimulates neutrophil responses, such as chemotactic migration, superoxide production, degranulation and aggregation (O'Flaherty *et al.*, 1981; Ingraham, *et al.*, 1982). PAF receptor is a plasma membrane protein that is coupled to guanine nucleotide binding proteins (Hwang, *et al.*, 1986). The binding of chemoattractants to receptors induces phosphoinositide hydrolysis by phospholipase C, promoting the formation of the intracellular messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG), which in turn cause the Ca^{2+} release from intracellular stores and the activation of protein kinase C, respectively (Nishizuka, 1984).

*To whom all correspondences should be addressed.

Protein kinase C may play a major role in the activation processes of neutrophils (Tauber, 1987). Protein kinase C is activated by DAG, phospholipids and Ca^{2+} (Castagna *et al.*, 1982). The Ca^{2+} induces protein kinase C to associate with the plasma membrane (Smolen *et al.*, 1981), while membrane DAG binds and then activates the adherent protein kinase C (O'Flaherty *et al.*, 1990). The activation of neutrophils by Ca^{2+}-insensitive protein kinase C has also been reported. Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, has been shown to stimulate aggregation, superoxide production, degranulation and phospholipid turnover without measurable change of cytosolic Ca^{2+} (Tauber, 1987). However, the inhibitor of protein kinase C, 1-(5-isoquinolinesulfonyl)-3-methyl piperazine (H-7) does not inhibit all neutrophil responses evoked by N-formylmethionyl-leucyl-phenylalanine (fMLP) (Gerard *et al.*, 1986; Berkow *et al.*, 1987). Thus, it is suggested that other protein kinases may also be involved in the signal transduction in neutrophils.

Neutrophils stimulated by fMLP or PMA show an increase in protein tyrosine phosphorylation (Berkow and Dodson, 1990). The inhibitors of protein tyrosine kinase, such as genistein and ST 638, inhibit both tyrosine phosphorylation and superoxide production (Berkow *et al.*, 1989; Tanimura *et al.*, 1992). These findings indicate that protein tyrosine phosphorylation is involved in the activation of neutrophil responses. It has been reported that superoxide production by the activation of protein kinase C in nonprimed neutrophils is stimulated by genistein and ST 638 (Tanimura *et al.*, 1992). The respiratory burst stimulated by the activation of protein kinase C might be affected by protein tyrosine kinase.

In this study, role of protein kinase C and protein tyrosine kinase in superoxide and H_2O_2 production and lysosomal enzyme release in PAF-stimulated neutrophils was investigated. Elevation of cytosolic Ca^{2+} level is an early event in the neutrophil responses to agonists, including fMLP and PAF (Westwick and Poll, 1986). However, role of these kinases in intracellular Ca^{2+} release and Ca^{2+} entry from the extracellular medium is uncertain. Their involvement in the elevation of cytosolic Ca^{2+} in the activated neutrophils were examined.

MATERIALS AND METHODS

L-α-Phosphatidylcholine, β-acetyl-γ-o-hexadecyl (PAF), phorbol 12-myristate 13-acetate (PMA), staurosporine, 1-(5-isoquinolinesulfon-yl)-3-methyl piperazine dihydrochloride (H-7), genistein, tyrphostin, methyl-2,5-dihydroxycinnamate, ferricytochrome c, scopoletin, o-dianisidine hydrochloride, diagnostic kit for acid phosphatase, cytochalasin B, fura-2/AM and Ficoll-Hypaque solution were purchased from Sigma Chemical Co.. All other reagents were of analytic grade.

Preparation of human neutrophils

Neutrophils were prepared from fresh whole human blood, anticoagulated with 10% acidcitrate-dextrose, by dextran sedimentation, hypotonic lysis of erythrocytes and Ficoll-Hypaque density centrifugation (Markert *et al.*, 1984). The neutrophils were suspended in Dulbecco's phosphate-buffered saline at a concentration of 1×10^7/ml. Final suspensions of neutrophils were comprised of about 97% neutrophils as judged from Wright-Giemsa stain and viability was more than 98% as judged from trypan blue dye exclusion.

After neutrophils were pretreated with cytochalasin B (5 μg/ml for 10^7 cells) for 5 min, the assay for the respiratory burst and degranulation was done.

Assay of superoxide production

The superoxide dependent reduction of ferricytochrome c was measured by the method of Markert *et al.* (1984). The reaction mixtures in plastic microfuge tubes contained 2×10^6 neutrophils, 75 μM ferricytochrome c, PAF, 20 mM HEPES-tris and Hanks' balanced salt solution (HBSS) buffer, pH 7.4 in a total volume of 1.0 ml. The reactions were performed in a 37℃ shaking water bath for 10 min. The reaction was then stopped by placing the tubes in melting ice, and the cells were rapidly pelleted by centrifuging at 1,500 g for 5 min at 4℃. The supernatants were taken, and the amount of reduced cytochrome c was measured at 550 nm.

The amount of reduced cytochrome c was calculated by using an extinction coefficient of 2.1 $\times 10^4$M-1 cm-1 at 550 nm (Cohen and Chovaniec, 1978).

Assay of hydrogen peroxide production

H_2O_2 produced from activated neutrophils was measured by change of scopoletin fluorescence. The reaction mixtures contained 2×10^6 neutrophils, 2.5 μM scopoletin, 5 μg/ml horse radish peroxidase, PAF, 20 mM HEPES-tris and HBSS buffer, pH 7.4 in a total volume of 1.0 ml. After preincubation of 5 min at 37℃ with inhibitors, the reaction was initiated by the addition of PAF. The decrease of scopoletin fluorescence by H_2O_2 produced was read at the wavelength of excitation, 343 nm and emission, 460 nm (Root *et al.*, 1975).

Assay of myeloperoxidase release

A 5×10^6/ml neutrophils in HBSS buffer with or without inhibitors were stimulated by adding PAF at 37℃. After 15 min of incubation, 250 μl of 0.2 M phosphate buffer, pH 6.2 and 250 μl of an equal mixture of 3.9 mM o-dianisidine HCl and 15 mM H_2O_2 were added. After 10 min of reincubation, the reaction was stopped by the addition of 250 μl of 1% sodium azide. The absorbance was read at 450 nm (Spangrude *et al.*, 1985).

Assay of acid phosphatase activity

Released amount of acid phosphatase from activated neutrophils was measured using Sigma diagnostic kit. The reaction mixtures contained 2×10^6 neutrophils, 1.0 μM PAF, 20 mM HEPES-tris and HBSS buffer, pH 7.4 in a total volume of 0.5 ml. After 15 min of incubation at 37℃, the reaction mixtures were centrifuged at 3,000 rpm for 10 min, and the supernatants were taken. Aliquotes (0.2 ml) were mixed with 0.5 ml of 4 mg/ml p-nitrophenyl phosphate disodium and 0.5 ml of 90 mM citrate buffer solution, pH 4.8. After 30 min of incubation at 37℃, the incubation was stopped by adding 5 ml of 0.1 N NaOH. The absorbance was read at 405 nm. Activity of acid phosphatase was estimated from the standard curve using p-nitrophenol standard solution and is expressed as the mUnit/2×10^6 cells.

Assay of cytosolic free calcium

Fura-2 loading and fluorescence measurement were performed by the method of Luscinskas *et al.* (1990). Neutrophils (approximately 5×10^7 cells/ml) were loaded with 2 mM fura-2/AM to 1 μM/10^7 cells at 37℃ for 10 min in the reaction mixtures contained HBSS buffer without calcium and magnesium (HBSS-CMF) and 20 mM HEPES-tris, pH 7.4. The suspension was then diluted 5 fold with 0.5% bovine serum albumin containing HBSS-CMF and was further incubated at 37℃ for 15 min. After loading, the suspension was centrifuged at 200 g for 10 min, and neutrophils were resuspended in 0.1% bovine serum albumin containing HBSS-CMF. This procedure was performed twice. Neutrophils were finally suspended in bovine serum albumin-free, HBSS-CMF as approximately 5×10^7 cells/ml. Fluorescence measurement was done with a Turner Spectrofluorometer (Model 430). Preloaded neutrophils (4×10^6) were suspended in the same reaction mixture in a final volume of 1.0 ml. After preincubation at 37℃ for 5 min with compounds, the response was initiated by the addition of PAF. The fluorescence change was read at an excitation wavelength of 340 nm and emission wavelength of 505 nm.

The traces on Ca^{2+} mobilization were representative of three experiments.

Assay of intracellular Ca^{2+} release

Intracellular Ca^{2+} release was measured by the modification of the method of Parys *et al.*, (1993) in Ca^{2+} free media containing 4×10^6/ml neutrophils (fura-2 loaded), 1 mM EGTA, 1 mM $MgCl_2$, HBSS and 20 mM HEPES-tris, pH 7.4 without extracellulary added Ca^{2+}. After 5 min of preincubation with or without inhibitors at 37℃, the Ca^{2+} release was initiated by adding PAF. The elevation of cytosolic Ca^{2+} was measured spectrofluorometrically.

Assay of Mn^{2+} influx

Influx of Mn^{2+} into cells was measured using the fura-2 fluorescence quenching technique (Demaurex *et al.*, 1992). Fura-2 loaded neutrophils (4×10^6/ml) were suspended in Ca^{2+}-and

Mg^{2+}-containing HBSS media. After 90 sec of stimulation with PAF, Mn^{2+} (0.5 mM) was added, and quenching of fura-2 fluorescence by Mn^{2+} influx was measured at an excitation wavelength of 360 nm and emission wavelength of 505 nm.

RESULTS

Inhibitory effects of staurosporine and genistein on superoxide production

The respiratory burst in cytochalasin B-treated neutrophils was stimulated by PAF. Amount of superoxide produced in neutrophils activated by 1.0 μM PAF was 4.80±0.66 nmol/10 min/2×10^6 cells (n=5). Role of protein kinase C and protein tyrosine kinase in the activation of the respiratory burst in PAF-activated neutrophils was investigated. The stimulatory effect of PAF on superoxide production was inhibited by 100 nM staurosporine and 50 μM H-7, inhibitors of protein kinase C and 10 μM genistein and 20 μM tyrphostin, inhibitors of protein tyrosine kinase (Fig. 1).

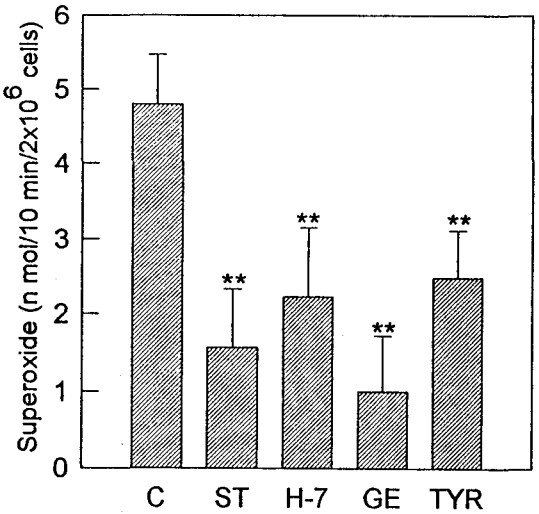

Fig. 1. Inhibitory effects of protein kinase inhibitors on superoxide production in neutrophil activated by PAF. Neutrophils(2×10^6 cells/ml) were stimulated with 1.0 μM PAF in the presence of inhibitors. Values are mean±S.D., n=5. C, no addition; ST, 100 nM staurosporine; H-7, 50 μM H-7; GE, 10 μM genistein; TYR, 20 μM H-7; GE, 10 μM genistein; TYR, 20 μM tyrphostin. **$p<0.01$ by Student's t-test.

H_2O_2 production, which is attained from the dismutation of $O_2^{\cdot-}$ (Fridovich, 1975), was measured by the oxidation of scopoletin. Oxidation of scopoletin by neutrophils was stimulated by PAF. Fig. 2 shows that 2 μM PAF-induced H_2O_2 production was inhibited by 100 nM staurosporine and 10 μM genistein.

Effects of staurosporine and genistein on lysosomal enzyme release

The secretion of lysosomal enzyme from activated neutrophils was assayed by measuring release of myeloperoxidase and acid phosphatase. Effects of kinase inhibitors on the release of lysosomal enzyme induced by PAF were examined. After neutrophils were preincubated for 5 min with the inhibitors, myeloperoxidase and acid phosphatase release was initiated by the addition of PAF. As shown in Fig. 3, 100 nM staurosporine, 50 μM H-7, 10 μM genistein and 20 μM tyrphostin inhibited the release of myeloperoxidase by 1.0 μM PAF. The stimulatory effect of PAF on acid phosphatase release

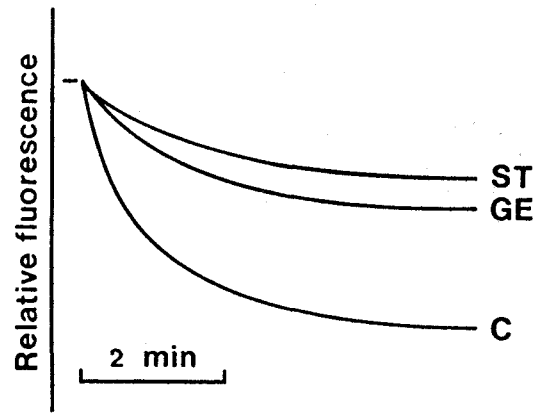

Fig. 2. Inhibition of PAF-induced hydrogen peroxide production by the kinase inhibitors. Neutrophils(2×10^6 cells/ml) were stimulated with 2 μM PAF in the presence of 100 nM staurosporine (ST) and 10 μM genistein (GE) or not (C). The traces are representative of three experiments.

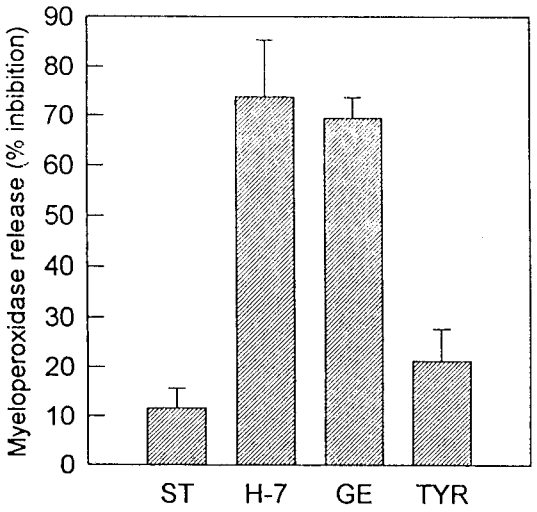

Fig. 3. Effects of protein kinase inhibitors on PAF-induced myeloperoxidase release. Neutorphils(5×10^6 cells/ml) were stimulated with 1.0 μM PAF for 15 min in the presence of inhibitors. Values are expressed as % inhibition and are means±S.D., n=5, ST, 100 nM staurosporine; H-7, 50 μM H-7; GE, 10 μM genistein; TYR, 20 μM tyrphostion.

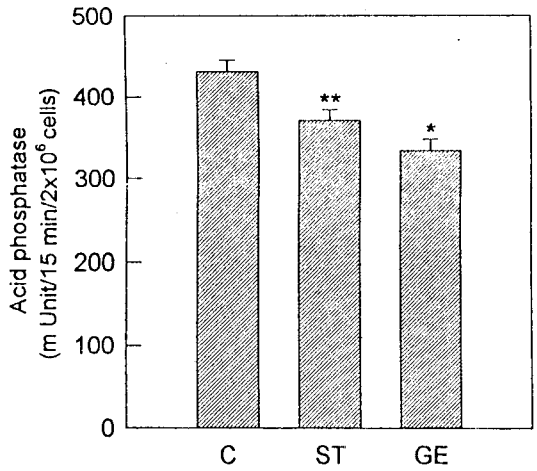

Fig. 4. Effects of staurosporine and genistein on acid phosphatase release by PAF. Neutrophils(2×10^6 cells/0.5 ml) were stimulated with 1.0 μM PAF in the presence of inhibitors. Values are mean± S.D., n=3. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein. **$p<0.01$, *$p<0.05$ by Student's t-test.

was also inhibited by staurosporine and genistein (Fig. 4).

Effects of staurosporine and genistein on calcium mobilization

The cytosolic Ca^{2+} level was assayed by measuring fluorescence change of fura-2 due to the complex formation of fura-2 and Ca^{2+}. One μM PAF elicited an increase of $[Ca^{2+}]_i$ in neutrophils. The maximum mobilization occured within 30 sec post addition, and then the level of $[Ca^{2+}]_i$ was maintained. Role of protein kinase C and protein tyrosine kinase in PAF-induced Ca^{2+} mobilization was examined. Fig. 5 shows that PAF-induced elevation of $[Ca^{2+}]_i$ was inhibited by 100 nM staurosporine, 10 μM genistein and 1 μg/ml methyl-2,5-dihydroxycinnamate (protein tyrosine kinase inhibitor).

The elevation of $[Ca^{2+}]_i$ is attained by both release of Ca^{2+} from intracellular stores and subsequent Ca^{2+} influx from the extracellular medium. In Ca^{2+} free media, the release of Ca^{2+} from the intracellular stores was induced by the addition of PAF. Influence of the kinase inhibitors on the intracellular Ca^{2+} release was observed. As can be seen in Fig. 6, 100 nM

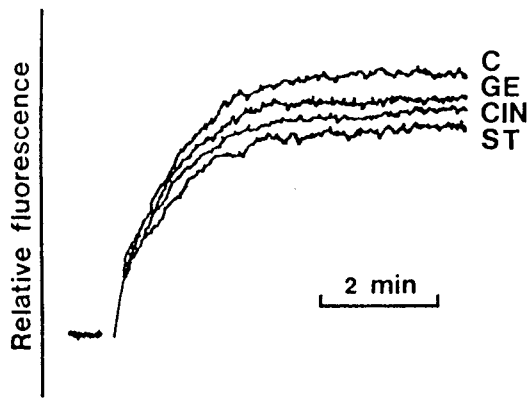

Fig. 5. Inhibition of PAF-induced elevation of $[Ca^{2+}]_i$ by staurosporine and genistein. Fura-2 loaded neutophils(4×10^6 cells/ml) were preincubated with inhibitors for 5 min, and then the response was initiated by the addition of 1.0 μM PAF. C, no addition; ST, 100 nM staurpsporine; GE, 10 μM genistein; CIN, 1 μg/ml methyl-25-dihydorxyxinnamate.

staurosporine inhibited the Ca^{2+} release in neutrophils activated by PAF, whereas the effects of genistein and methyl-2,5-dihydroxycinnamate were not detected.

The activity of the Ca^{2+} influx pathway was assayed with Mn^{2+} influx. The Mn^{2+} is considered to permeate through the neutrophil Ca^{2+} influx pathway activated by chemoattractants (Demaurex *et al.*, 1992). The experiments were done at an excitation wavelength of 360 nm. In this wavelength, PAF did not cause fluorescence change in Fura-2-loaded neutrophils. When added to 1.0 μM PAF-stimulated neutrophils, 0.5 mM Mn^{2+} caused a rapid and continuous decrease in fluorescence. The stimulated increase of Mn^{2+} influx was inhibited by 100 nM staurosporine, 10 μM genistein and 1 μg/ml methyl-2,5-dihydroxycinnamate (Fig. 7).

It has been suggested that activation of PKC with PMA decreases agonist-induced elevation of $[Ca^{2+}]_i$ by inhibition of Ca^{2+} mobilization (MaCarthy *et al.*, 1989). The activation of protein kinase C is thought to decrease agonist-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils. As shown in Fig. 8, after the pretreatment with 0.1 μg/ml PMA, the stimulatory effect of 1.0 μM

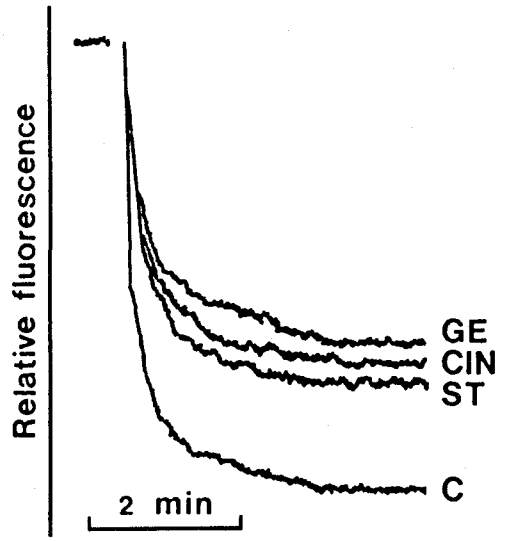

Fig. 7. Effects of staurosporine and genistein on Mn^{2+} influx. Fura-2 loaded neutrophils(4×10^6 cells/ml) were stimulated with 1.0 μM PAF for 90 sec in the presence of inhibitors, and then Mn^{2+} influx was initiated by adding 0.5 mM Mn^{2+}. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; CIN, 1 μg/ml methyl-2,5-dihydroxycinnamate.

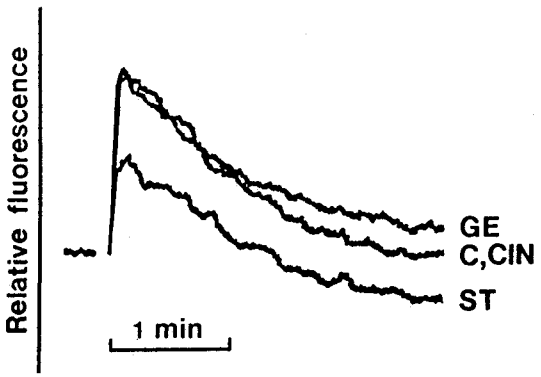

Fig. 6. Effects of the kinase inhibitors on the intracellular Ca^{2+} release. In Ca^{2+} free media, fura-2 loaded neutrophils (4×10^6 cells/ml) were stimulated to induce Ca^{2+} release with 1.0 μM PAF. C, no addition; ST, 100 nM staurosoporine; GE, 10 μM genistein; CIN, 1 μg/ml methyl-2,5-dihydroxycinnamate.

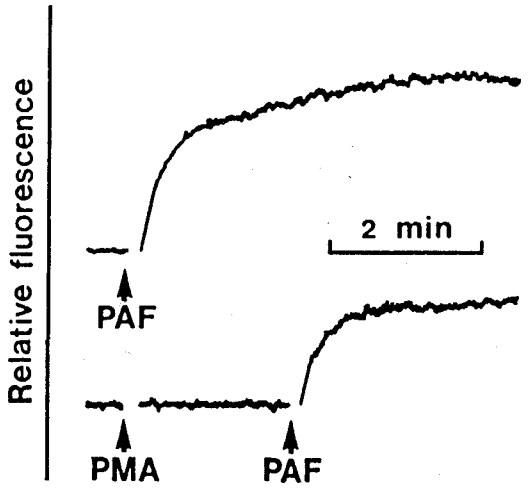

Fig. 8. Inhibition of PAF-induced elevation of $[Ca^{2+}]_i$ by pretreatment of PMA. Fura-2 loaded neutorphils (4×10^6 cells ml) was treated with 0.1 μg/ml PMA, and then 1.0 μM PAF was added to induce elevation of $[Ca^{2+}]_i$ sequentially.

PAF on the elevation of neutrophil cytosolic Ca^{2+} was significantly decreased.

DISCUSSION

Activation of neutrophils by chemoattractants, such as PAF and fMLP initiates ionic and molecular changes including membrane depolarization (Mottola and Romeo, 1982), phospholipid inositol turnover (Bareis *et al.*, 1982) and elevation of intracellular Ca^{2+} (Westwick and Poll, 1986), and these changes are followed by responses, respiratory burst and degranulation. Chemoattractants appear to exert their action by binding to G protein-linked, cell surface receptors at the plasma membrane (Verghese *et al.*, 1985). Stimulation of receptors causes the activation of phospholipase C and A_2, which promotes the formation of InsP3, DAG and arachidonate (Kawaguchi and Yasuda, 1986; Berridge, 1987). In addition, the binding of PAF to the receptors is known to induce the activation of tyrosine kinase (Gomez-Cambronero *et al.*, 1991). $InsP_3$ increases intracellular Ca^{2+} through the promotion of intracellular Ca^{2+} release (Nishizuka, 1984), and DAG activates the Ca^{2+}-adherent protein kinase C (O'Flaherty *et al.*, 1990).

Elevation of intracellular Ca^{2+} level, activation of protein kinase C and phosphorylaton of tyrosine kinase appear to regulate neutrophil responses, degranulation and superoxide production. In PAF-activated neutrophils, superoxide and H_2O_2 production and myeloperoxidase and acid phosphatase release were inhibited by staurosporine, H-7, genistein and tyrphostin. These findings indicate that protein kinase C and protein tyrosine kinase may be involved in the activation of neutrophil responses by PAF. The weak inhibitory effect of kinase inhibitors on acid phosphatase release suggests that the process of degranulation is regulated by other systems chiefly rather than the kinases. The activation mechanism of the respiratory burst may be different from the lysosomal enzyme release (Shin *et al.*, 1989).

Surface stimulation by particulate or soluble agents leads to the elevation of $[Ca^{2+}]_i$ in neutrophils (Goldstein *et al.*, 1975). A rise in cytosolic Ca^{2+} level is thought to play an important role in the activation of neutrophil responses. The elevation of $[Ca^{2+}]_i$ is attained by both release of Ca^{2+} from the intracellular stores and Ca^{2+} influx from the extracellular medium (Pozzan *et al.*, 1983; Westwick and Poll, 1986). The release of Ca^{2+} from the intracellular stores is mediated by $InsP_3$. The $InsP_3$ activates specific Ca^{2+} channels localized in the membrane of intracellular stores (Berridge, 1993). The channels appear to be regulated by Ca^{2+}, ATP and probably protein kinases (Zhang *et al.*, 1993). Intracellular Ca^{2+} level of neutrophils was significantly increased by the addition of PAF. Role of protein kinase C and protein tyrosine kinase in the elevation of $[Ca^{2+}]_i$ in neutrophils activated by PAF was investigated. Staurosporine, genistein and methyl-2,5-dihydroxycinnamate inhibited the elevation of $[Ca^{2+}]_i$ by PAF. Thus, PAF-induced elevation of $[Ca^{2+}]_i$ may be regulated by protein kinase C and protein tyrosine kinase.

In Ca^{2+} free media, PAF-induced release of Ca^{2+} from the intracellular stores was inhibited by staurosporine but was not affected by genistein and methyl-2,5-dihydroxycinnamate. These findings indicate that protein kinase C could affect the intracellular Ca^{2+} release in PAF-activated neutrophils. And it is unlikely that the Ca^{2+} release is affected by the protein tyrosine phosphorylation. The regulatory mechanism involved in Ca^{2+} entry across the plasma membrane of neutrophils has not been clearly elucidated. It is reported that Ca^{2+} influx in granulocytes may be not accomplished by voltage-operated, receptor-operated or second messenger-operated Ca^{2+} channels (Jaconi *et al.*, 1993). The Ca^{2+} entry in platelets is probably controlled by tyrosine kinases (Sargeant *et al.*, 1993). The divalent cation Mn^{2+} has been shown to permeate through the neutrophil Ca^{2+} influx pathway activated by chemoattractants (Demaurex *et al.*, 1992; Jaconi *et al.*, 1993). However, role of the kinases in Ca^{2+} influx is uncertain. In PAF-activated neutrophils, Mn^{2+} influx was inhibited by staurosporine, genistein and methyl-2,5-dihydroxycinnamate. The involvement of protein kinase C and protein tyrosine kinase in the control of Ca^{2+} entry in

PAF-activated neutrophils is suggested. Protein kinase C and protein tyrosine kinase may be involved in the elevation of $[Ca^{2+}]_i$ in PAF-stimulated neutrophils by their different regulatory role in intracellular Ca^{2+} release and Ca^{2+} influx.

The activation of protein kinase C can inhibit agonist-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils by blocking stimulation of influx of divalent cations from the extracellular medium and by partially inhibiting release of Ca^{2+} from the intracellular stores (McCarthy *et al.*, 1989). After neutrophils were pretreated with PMA, an activator of protein kinase C, the stimulatory effect of PAF on Ca^{2+} mobilization was significantly reduced. The result supports the above view that agonist-induced stimulation of the response could be attenuated by the activation of protein kinase C.

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=국문초록=

Protein Kinase 억제제 첨가 후 Platelet-Activating Factor에 의하여 자극된 호중구반응의 변경

중앙대학교 의과대학 약리학교실

이강건 · 고지영 · 함동석 · 신용규 · 이정수

Platelet-activating factor (PAF)에 의하여 자극된 호중구 respiratory burst, 탈과립과 세포질 칼슘농도의 증가에 있어 protein kinase C와 protein tyrosine kinase의 역할을 관찰하였다.

PAF에 의하여 자극된 호중구에서 superoxide 및 H_2O_2의 생성과 myeloperoxidase와 acid phosphatase의 유리는 protein kinase C 억제제인 staurosporine과 H-7 그리고 protein tyrosine kinase 억제제인 genistein과 tyrphostin에 의하여 억제되었다. PAF에 의한 호중구 세포내 칼슘농도의 증가는 staurosporine, genistein과 methyl-2,5-dihydroxycinnamate에 의하여 억제되었다. Staurosporine은 PAF에 의하여 자극된 호중구에서 세포내 칼슘유리와 망간유입을 억제하였다. Genistein과 methyl-2,5-dihydroxycinnamate는 PAF에 의한 망간유입을 억제하였으나, 세포내 칼슘유리에 대한 이들의 효과는 관찰되지 않았다. PMA에 의하여 활성화된 호중구에서 세포내 칼슘농도의 증가에 대한 PAF의 자극효과는 감소되었다.

Protein kinase C와 protein tyrosine kinase는 PAF에 의하여 자극된 호중구에서의 respiratory burst, lysosomal enzyme유리와 칼슘동원에 관여할 것으로 제시된다. 세포내 칼슘농도의 증가는 protein kinase의 영향을 다르게 받는 세포내 칼슘유리와 세포외부로 부터의 칼슘유입에 의하여 이루어질 것으로 추정된다. Protein kinase C가 활성화되어 있는 상태에서 세포내 칼슘동원에 대한 PAF의 자극작용은 감소될 것으로 시사된다.