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Effects of protein kinase inhibitors on the stimulated neutrophil responses by degraded immunoglobulin G

Sang K. Ahn, Yong K. Shin, Hee J. Kang, Eun S. Han, Chung S. Lee *

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

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Abstract

Effects of protein kinase C inhibitors, staurosporine and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride and protein tyrosine kinase inhibitors, genistein, tyrphostin and 2,5-dimethylcinnamate on the neutrophil responses stimulated by immunoglobulin G (IgG), complement C5a or platelet-activating factor were studied. After receptor binding, the role of protein kinase C and protein tyrosine kinase in the stimulation of neutrophil responses, superoxide production and lysosomal enzyme release in degraded IgG-activated neutrophils may be similar to chemoattractant-stimulated cells. In contrast to complement C5a or platelet-activating factor, protein tyrosine kinase appears to play an important role in the regulation of intracellular Ca^{2+} mobilization in neutrophils activated by degraded IgG rather than by protein kinase C.

Keywords: Protein kinase inhibitor; Respiratory burst; Degranulation; Ca^{2+} mobilization; Neutrophil, human

1. Introduction

Neutrophils liberate reactive oxygen species (Babior, 1978), lysosomal enzymes (Zurier et al., 1974) and lipid-derived products, such as leukotrienes (Palmer and Salmon, 1983) and platelet-activating factor (PAF) (Jouvin-Marche et al., 1984) when stimulated by particulate and soluble stimuli, and they play an important role in host defense mechanisms and inflammatory responses (Fantone and Ward, 1982). Stimulation of the respiratory burst produces superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and probably hydroxyl radical ($OH^\cdot$) (Babior, 1978). Protein kinase C appears to play a major role in signal transduction and other cellular processes (Nishizuka, 1984). Protein kinase C could be activated by 1,2-diacylglycerol, phospholipids and Ca^{2+} (Castagna et al., 1982). Protein kinase C is considered to be involved in the activation of the respiratory burst. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, has been shown to stimulate neutrophils and cause aggregation, stimulation of the respiratory burst, hydrolysis of membrane phospholipid and degranulation of specific granules (Kramer et al., 1984; Tauber, 1987). PMA is known to activate protein kinase C directly without involving membrane receptor binding.

The inhibitors of protein kinase C fail to inhibit stimulation of neutrophil responses, respiratory burst and degranulation by formyl-methionyl-leucyl-phenylalanine (fMLP) (Sha'afi et al., 1988) and rather stimulate superoxide production in granulocyte-macrophage colony stimulating factor (GM-CSF)-primed neutrophils (Tanimura et al., 1992). These findings indicate that the activation of other protein kinases may be involved in the activation of neutrophils. On the other hand, an inhibitor of protein tyrosine kinase inhibits respiratory burst (Berkow et al., 1989) and production of eicosanoids, prostaglandin E_2 (Glaser et al., 1993) in phagocytic cells activated by a variety of agonists.

Phagocytosis of degraded immunoglobulin G (IgG) by neutrophils leads to stimulation of the respiratory burst, release of lysosomal enzymes (Fantone and Ward, 1982) and elevation of intracellular Ca^{2+} (Young et al., 1984). However, the activation processes involved in the stimulation of neutrophil responses following the binding Fc receptors, are not clearly defined. This study was done to examine the role of protein kinase C and protein tyrosine kinase in the stimulation of neutrophil responses by degraded IgG. The effects of protein kinase inhibitors on the stimulatory action of degraded IgG were compared with their effects on C5a and platelet-activating factor.

* Corresponding author. Tel.: (02) 820-5659; fax: 82-2-813-5387.

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2. Materials and methods

2.1. Preparation of human neutrophils

Neutrophils were prepared from fresh whole human blood, anticoagulated with 10% acid-citrate-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 (PBS), pH 7.4 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 by trypan blue dye exclusion.

Human IgG was heated to degrade at 63°C for 30 min. 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.

2.2. 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*, degraded IgG, 20 mM Hepes-Tris and Hanks' balanced salt solution (HBSS), pH 7.4, in a total volume of 1.0 ml. The reactions were performed in a 37°C shaking water bath for 15 min. The reaction was then stopped by placing the tubes in melting ice, and the cells were rapidly pelleted by centrifuging at $800 \times g$ for 5 min at 4°C. 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 2.1×10^4 M^{-1} cm^{-1} at 550 nm (Cohen and Chovaniec, 1978).

2.3. Assay of hydrogen peroxide production

H_2O_2 produced from activated neutrophils was measured by a change in scopoletin fluorescence. The reaction mixtures contained 2×10^6 neutrophils, 2.5 μM scopoletin, 5 μg/ml horseradish peroxidase, degraded IgG, 20 mM Hepes-Tris and HBSS buffer, pH 7.4 in a total volume of 1.0 ml. After 5 min of preincubation at 37°C with compounds, the reaction was initiated by the addition of agonist. The decrease in scopoletin fluorescence by H_2O_2 produced was read at the wavelength of excitation, 343 nm and emission, 460 nm (Root et al., 1975).

2.4. Assay of acid phosphatase activity

The released amount of acid phosphatase from activated neutrophils was measured using a Sigma diagnostic kit. The reaction mixtures contained 2×10^6 neutrophils, degraded IgG, HBSS, pH 7.4 in a total volume of 0.5 ml. After 15 min of incubation at 37°C, the reaction mixtures were centrifuged at $800 \times g$ for 5 min, and the supernatants were separated. Aliquots (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°C, the incubation was stopped by adding 5 ml of 0.1 N NaOH. The absorbance was read at 405 nm. The activity of acid phosphatase was estimated from the standard curve using *p*-nitrophenol standard solution and is expressed as mU/2×10^6 cells.

2.5. Assay of myeloperoxidase release

Neutrophils (5×10^6/ml) in HBSS buffer with or without inhibitors were stimulated by adding degraded IgG. 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 of cells containing reaction mixtures was read at 450 nm (Spangrude et al., 1985).

2.6. Assay of cytosolic free calcium

Fura-2 loading and measurement of fluorescence and Ca^{2+} content 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°C for 10 min in the reaction mixtures containing 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 further incubated at 37°C for 15 min. After loading, the suspension was centrifuged at $200 \times g$ 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 at approximately 5×10^7 cells/ml. Fluorescence measurement was done with a Turner spectrofluorometer (Model 430). Preloaded neutrophils (4×10^6) were suspended in Ca^{2+}- and Mg^{2+}-containing HBSS media in a final volume of 1.0 ml. After preincubation at 37°C for 5 min with compounds, the response was initiated by the addition of agonist. The fluorescence change was read at an excitation wavelength of 340 nm and emission wavelength of 510 nm.

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

2.7. Drugs

Human IgG, complement C5a, L-α-phosphatidylcholine-β-acetyl-*o*-hexadecyl, staurosporine, 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine dihydrochloride (H-7), genistein, tyrphostin, 2,5-dimethylcinnamate, per-

tussis toxin, verapamil, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), ferricytochrome c, scopoletin, diagnostic kit for acid phosphatase, o-dianisidine, fura-2/AM were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of analytical reagent grade.

2.8. Data analysis

The results obtained in various experiments were analysed for level of significance using the Student's t test.

3. Results

3.1. Effects of protein kinase inhibitors on superoxide production in activated neutrophils

The respiratory burst was stimulated by degraded IgG, C5a and platelet-activating factor. A 0.5 mg/ml degraded IgG stimulated superoxide production in human neutrophils, and 24.50 ± 0.54 nmol/15 min/10^6 cells of superoxide anion ($n = 4$) was produced. The role of protein kinase C and protein tyrosine kinase in activation of neutrophils by degraded IgG was investigated. In this study, the concentrations of kinase are the doses used in previously reported data. As shown in Fig. 1, degraded IgG-induced superoxide production was inhibited by protein kinase C inhibitors, 100 nM staurosporine and 50 μM H-7 and protein tyrosine kinase inhibitors, 10 μM genistein and 20 μM tyrphostin, and an inhibition of 53%, 23%, 42% and 26% respectively occurred. Staurosporine at 100 nM and 10 μM genistein inhibited superoxide production in neutrophils activated by either 20 nM C5a or 1 μM platelet-activating factor (Fig. 2). The inhibitory effects of both inhibitors on the chemoattractant's effects were similar to the effect of degraded IgG.

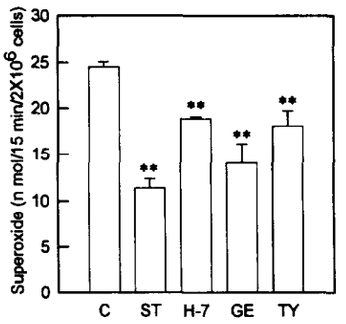

Fig. 1. Effects of protein kinase inhibitors on superoxide production in neutrophils activated by degraded IgG. Neutrophils were stimulated with 0.5 mg/ml degraded IgG in the presence of inhibitors. Values are means $\pm$ S.D., $n = 4$. C: no addition; ST: 100 nM staurosporine; H-7: 50 μM H-7; GE: 10 μM genistein; TY: 20 μM tyrphostin. * * $P < 0.01$ by Student's t test.

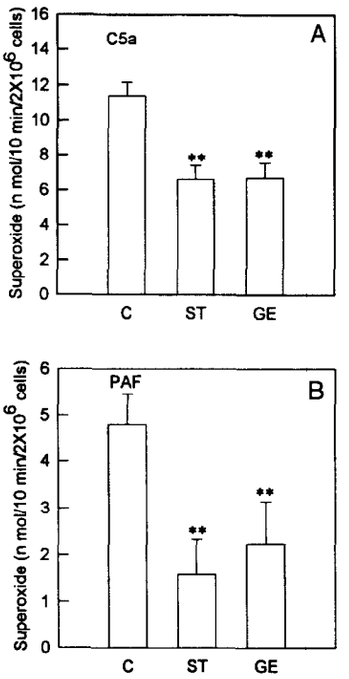

Fig. 2. Inhibitory effects of staurosporine and genistein on C5a- or PAF-induced superoxide production. Neutrophils were stimulated with 20 nM C5a (or 1.0 μM PAF) in the presence of inhibitors. Values are means $\pm$ S.D., $n = 4$–5. C: no addition; ST: 100 nM staurosporine; GE: 10 μM genistein. * * $P < 0.01$ by Student's t test.

3.2. Effects of staurosporine and genistein on H_2O_2 production

H_2O_2 production, which is attained from the dismutation of O_2^- (Fridovich, 1975), was measured by the oxidation of scopoletin. Oxidation of scopoletin by neutrophils was stimulated by degraded IgG, C5a and platelet-activating factor. Effects of kinase inhibitors on H_2O_2 production was examined. Fig. 3 shows that 50 μM H-7 and 100 nM staurosporine inhibited H_2O_2 production in neutrophils activated by 0.5 mg/ml degraded IgG, whereas 10 μM

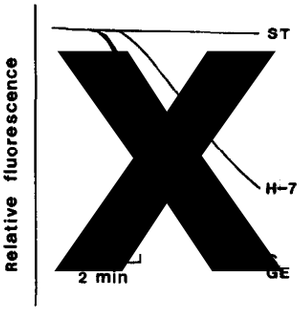

Fig. 3. Inhibition of degraded IgG-induced H_2O_2 production by staurosporine. Neutrophils (2×10^6 cells/ml) were stimulated with 0.25 mg/ml degraded IgG in the presence of inhibitors. C: no addition; ST: 100 nM staurosporine; H-7: 50 μM H-7; GE: 10 μM genistein.

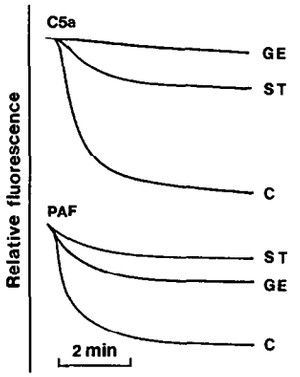

Fig. 4. Effects of staurosporine and genistein on C5a- or PAF-induced H_2O_2 production. Neutrophils (2×10^6 cells/ml) were stimulated with 20 nM C5a or 2 μM PAF in the presence of inhibitors. C: no addition; ST: 100 nM staurosporine; GE: 10 μM genistein.

genistein did not show inhibitory effect. A 20 nM C5a- and 2 μM platelet-activating factor-induced H_2O_2 production was inhibited by 100 nM staurosporine and 10 μM genistein (Fig. 4). In contrast to chemoattractants, H_2O_2 production induced by degraded IgG did not respond to the inhibition of protein tyrosine kinase.

3.3. Effects of protein kinase inhibitors on lysosomal enzyme release

The secretion of lysosomal enzymes from neutrophils was assayed by measuring the release of acid phosphatase and myeloperoxidase. The release of acid phosphatase and myeloperoxidase was induced by degraded IgG. Fig. 5 shows that 100 nM staurosporine, 50 μM H-7, 10 μM genistein and protein tyrosine kinase inhibitors, 1 μg/ml 2,5-dimethylcinnamate inhibited lysosomal enzyme release by 0.5 mg/ml degraded IgG. C5a at 20 nM and 1 μM platelet-activating factor also stimulated the release of acid phosphatase, and the stimulatory effect of C5a and platelet-activating factor was inhibited by 100 nM staurosporine and 10 μM genistein while staurosporine has a little inhibitory effect on C5a compared with platelet-activating factor (Fig. 6). The inhibitory pattern of both inhibitors on degraded IgG-induced acid phosphatase release was similar to that of platelet-activating factor.

3.4. Effects of staurosporine and genistein on intracellular calcium level

The $[Ca^{2+}]_i$ was measured with the increase in fluorescence due to the complex formation of an increased Ca^{2+} with fura-2. Degraded IgG at 0.5 mg/ml caused an increase in intracellular Ca^{2+} level ($[Ca^{2+}]_i$) in fura-2-loaded neutrophils in 1.23 mM Ca^{2+}-containing medium. The $[Ca^{2+}]_i$ rose to a maximum within 15 s post addition, and then the $[Ca^{2+}]_i$ was gradually decreased to the resting level over the subsequent several minutes (Fig. 7). The role of protein kinase C and protein tyrosine kinase in

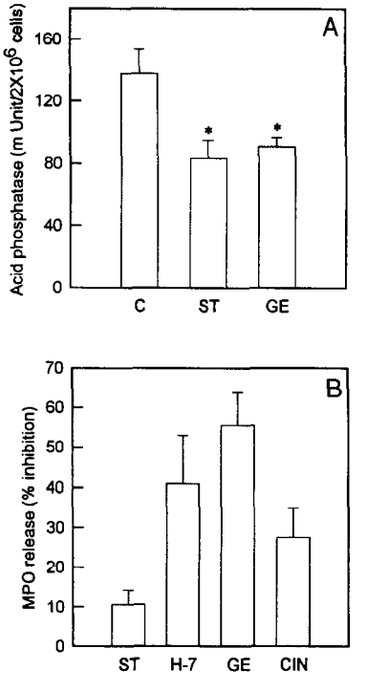

Fig. 5. Inhibitory effects of staurosporine and genistein on lysosomal enzyme release by degraded IgG. Neutrophils were stimulated with 0.5 mg/ml degraded IgG in the presence of inhibitors for 15 min. A degraded IgG-stimulated neutrophils showed an absorbance change of 0.464 ± 0.031 in myeloperoxidase (MPO) release. Values are means $\pm$ S.D., $n=4$. C: no addition; ST: 100 nM staurosporine; H-7: 50 μM H-7; GE: 10 μM genistein; CIN: 1 μg/ml 2,5-dimethylcinnamate. * $P<0.05$ by Student's t test.

degraded IgG-induced Ca^{2+} mobilization was examined. A 0.5 mg/ml degraded IgG-induced elevation of $[Ca^{2+}]_i$ was inhibited by 10 μM genistein but was not inhibited by 100 nM staurosorine. Verapamil, a Ca^{2+} channel blocker at 0.1 mM, and 5 mM EGTA, a Ca^{2+} chelator, inhibited the elevation of $[Ca^{2+}]_i$ by degraded IgG. As shown Fig. 8, 100 nM staurosporine, 50 μM H-7, 10 μM genistein and 1 μg/ml 2,5-dimethylcinnamate inhibited elevation of $[Ca^{2+}]_i$ by either 20 nM C5a or 1.0 μM platelet-activating

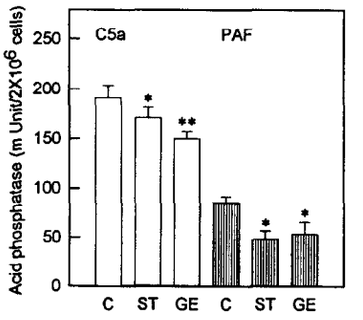

Fig. 6. Inhibition of C5a- or PAF-induced acid phosphatase release by staurosporine and genistein. Neutrophils (2×10^6 cells/0.5 ml) were stimulated with 20 nM C5a and (or 1.0 μM PAF) in the presence of inhibitors for 15 min. Values are means $\pm$ S.D., $n=3$–4. C: no addition; ST: 100 nM staurosporine; GE: 10 μM genistein. ** $P<0.01$, * $P<0.05$ by Student's t test.

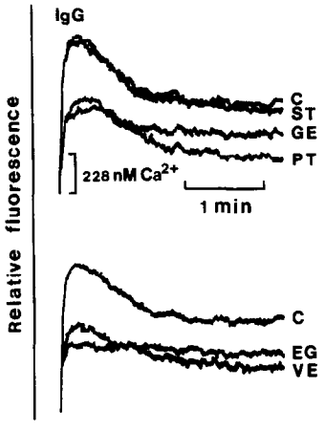

Fig. 7. Inhibitory effect of genistein on degraded IgG-induced elevation of $[Ca^{2+}]_i$. Changes of $[Ca^{2+}]_i$ in stimulated neutrophils (4×10^6 cells/ml) were measured as a fluorescence change of fura-2. After 5 min of preincubation with inhibitors, the response was initiated by the addition of 0.5 mg/ml degraded IgG. C: no addition; ST: 100 nM staurosporine; GE: 10 μM genistein; PT: 0.1 μg/ml pertussis toxin; EG: 5 mM EGTA; VE: 0.1 mM verapamil.

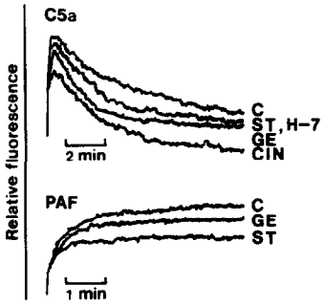

Fig. 8. Inhibition of agonist-induced elevation of $[Ca^{2+}]_i$ by staurosporine and genistein. Neutrophils (4×10^6 cells/ml) were preincubated with inhibitors for 5 min, and then the response was initiated by the adding 20 nM C5a (or 1.0 μM PAF). C: no addition; ST: 100 nM staurosporine; H-7: 50 μM H-7; GE: 10 μM genistein; CIN: 1 μg/ml 2,5-dimethylcinnamate.

factor. Thus, in contrast to chemoattractants, the intracellular Ca^{2+} mobilization does not appear to be regulated by protein kinase C system.

4. Discussion

Chemoattractants and immune complexes appear to exert their action by binding to protein G-linked cell surface receptors at the plasma membrane (Hwang, 1988; Siciliano et al., 1990). Stimulation of these receptors causes activation of phospholipases C and A_2, promoting the formation of inositol 1,4,5-trisphosphate, 1,2-diacylglycerol and arachidonate (Kawaguchi and Yasuda, 1986; Berridge, 1987). These mediators are responsible for the release of Ca^{2+} from intracellular stores and the activation of protein kinase C. A rise in cytosolic Ca^{2+} concentration is considered to be an important factor in the stimulation of neutrophil responses. Elevated $[Ca^{2+}]_i$ induces translocation of protein kinase C to the plasma membrane (Smolen et al., 1981), and the Ca^{2+}-adherent protein kinase C is activated by 1,2-diacylglycerol located at the membrane (O'Flaherty et al., 1990). In addition, receptor binding also translocates cytosolic proteins, p47-*phox*, p67-*phox* and GTP-binding protein, Rac to the plasma membrane (Babior, 1992; Quinn et al., 1993). They assemble in a complex that actively phosphorylates response-eliciting proteins and activate superoxide forming NADPH oxidase.

Degraded IgG is considered as an inflammatory mediator. In inflammatory joints, phagocytosis of degraded IgG leads to the activation neutrophils and may cause tissue damage by the release of lysosomal enzymes and oxidants (Hannestad, 1967). However, the activation processes involved in the stimulation of neutrophil responses after the binding of Fc receptors by IgG have not been elucidated clearly. Staurosporine, H-7, genistein and tyrphostin inhibited superoxide and H_2O_2 production in neutrophils activated by degraded IgG. The stimulatory effects of C5a and platelet-activating factor on superoxide and H_2O_2 production was inhibited by staurosporine and genistein. Thus, protein kinase C and protein tyrosine kinase may be involved in the stimulation of respiratory burst in neutrophils by degraded IgG, C5a and platelet-activating factor. However, unlike C5a and platelet-activating factor, the stimulatory effect of degraded IgG on H_2O_2 production was not affected by genistein. This discrepancy indicates that in contrast to chemoattractants, the stimulatory effect of degraded IgG on H_2O_2 production may not respond to the inhibition of protein tyrosine kinase. The regulatory role of protein kinase C and protein tyrosine kinase in degraded IgG-, C5a- or platelet-activating factor-induced release of acid phosphatase was also suggested, and the inhibitory pattern of protein kinase inhibitors on acid phosphatase release induced by degraded IgG was similar to that of platelet-activating factor. However, the involvement of protein kinase C in the lysosomal enzyme release from activated neutrophils by C5a is uncertain.

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+} is considered to play an important role in the stimulation of neutrophil responses. The rise in $[Ca^{2+}]_i$ is attained by both the release of Ca^{2+} from intracellular stores and Ca^{2+} influx across the plasma membrane (Pozzan et al., 1983; Westwick and Poll, 1986). It has been demonstrated that the release of Ca^{2+} from intracellular stores is mediated by inositol 1,4,5-trisphosphate (Nishizuka, 1984). Degraded IgG- or platelet-activating factor-induced elevation of $[Ca^{2+}]_i$ was inhibited by EGTA and verapamil (data on platelet-activating factor are not shown). These results partly support the above suggestion on intracellular Ca^{2+} mobilization. The role of protein kinase C and protein tyrosine kinase in degraded IgG-induced Ca^{2+} mobilization may be different from that in Ca^{2+} mobilization induced by C5a and platelet-activating factor. Genistein and pertussis toxin in-

hibited elevation of $[Ca^{2+}]_i$ by degraded IgG, whereas staurosporine did not affect it. Degraded IgG-induced elevation of $[Ca^{2+}]_i$ also was not inhibited by H-7 in our other experiments (data not shown). Thus, degraded IgG-induced intracellular Ca^{2+} mobilization does not appear to be regulated by protein kinase C. In contrast, C5a and platelet-activating factor-induced Ca^{2+} mobilization was inhibited by protein kinase C inhibitor. Effects of staurosporine and H-7 on degraded IgG-elicited elevation of $[Ca^{2+}]_i$ were different from their effects on superoxide production. These findings probably support that the respiratory burst could also be stimulated by Ca^{2+}-independent activation mechanism.

After receptor bindings, the role of protein kinase C and protein tyrosine kinase in the stimulation of neutrophil responses, respiratory burst and degranulation in degraded IgG-activated neutrophils may be similar to that in chemoattractants-stimulated cells. In contrast to C5a or platelet-activating factor, protein tyrosine kinase appears to play an important role in the regulation of intracellular Ca^{2+} mobilization in neutrophils activated by degraded IgG rather than by protein kinase C.

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