Korean J. of Pharmacology
Vol. 32, No. 3, pp. 417~424, 1996

# Regulatory Action of Protein Tyrosine Kinase in Intracellular Calcium Mobilization in C5a-stimulated Neutrophils

Won-Tae Choi, Eun-Sook Han and Chung-Soo Lee*

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

## ABSTRACT

The present study was done to examine the involvement of protein kinase C and protein tyrosine kinase in intracellular $Ca^{2+}$ mobilization in C5a-stimulated neutrophils. Although protein kinase C inhibitors, staurosporine and H-7 inhibited intracellular $Ca^{2+}$ release in C5a-stimulated neutrophils, they did not affect $Ca^{2+}$ influx across the plasma membrane and elevation of $[Ca^{2+}]_i$. C5a-induced intracellular $Ca^{2+}$ release and $Ca^{2+}$ influx were inhibited by protein tyrosine kinase inhibitors, genistein and methyl-2,5-dihydroxycinnamate. ADP-evoked elevation of $[Ca^{2+}]_i$ was inhibited by genistein and methyl-2,5-dihydroxycinnamate but was not affectd by staurosporine and H-7. Genistein and methyl-2,5-dihydroxycinnamate reduced the store-regulated $Ca^{2+}$ influx in thapsigargin-treated neutrophils, while the effect of staurosporine and H-7 was not detected. When neutrophils were preincubated wih phorbol 12-myristate 13-acetate, the stimulatory effect of C5a on the elevation of $[Ca^{2+}]_i$ was reduced. These results suggest that protein tyrosine kinase may be involved in control of intracellular $Ca^{2+}$ release and $Ca^{2+}$ influx across the plasma membrane in C5a-activated neutrophils.

**Key Words:** C5a, Protein kinase inhibitors, Intracellular calcium mobilization, Neutrophils

## INTRODUCTION

Neutrophils exposure to external stimulating agents result in molecular and functional changes, including phospholipid inositol turnover (Gil *et al.*, 1982), elevation of cytosolic $Ca^{2+}$ (Westwick and Poll, 1986) and tyrosine phosphorylation (Berkow and Dodson, 1990), and these changes are followed by responses, superoxide production and degranulation (Fantone and Ward, 1982). These released reactive oxygen species and lysosomal enzymes play an important role in host defence mechanisms and inflammatory responses.

Increased intracellular $Ca^{2+}$ may involve in the activation of neutrophil responses including degranulation due to surface stimulation. The rise in $[Ca^{2+}]_i$ is attained by both 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 InsP3 (Dougherty *et al.*, 1984; Krause *et al.*, 1985). However, the mechanism, which is involved in $Ca^{2+}$ influx, has not been elucidated clearly (Putney, 1986). It has been shown that $Ca^{2+}$ influx in granulocytes does not appear to involve

*To whom all correspondences should be addressed.

voltage-operated, receptor-operated, or second messenger-operated $Ca^{2+}$ channels. Inositol-(1,3,4,5)tetrakisphosphate ($InsP_4$) has been reported to be implicated in $Ca^{2+}$ influx (Irvine and Moor, 1986; Lückhoff and Clapham, 1992). On the other hand, the involvement of protein kinases in intracellular $Ca^{2+}$ mobilization are uncertain.

Complement C5a is a potent chemotaxin for neutrophils and macrophages (Goldstein, 1992). It stimulates these cells to produce superoxide anion, to release lysosomal enzymes and alters their surface properties leading to enhanced adhesion and aggregation. The activation processes, which transduces response changes after C5a receptors binding, appear to involve pertussis toxin-sensitive G proteins (Becker *et al.*, 1985).

The present study was done to examine the involvement of protein kinase C and protein tyrosine kinase in intracellular $Ca^{2+}$ elevation, intracellular $Ca^{2+}$ release and $Ca^{2+}$ influx from the extracellular medium in C5a-stimulated neutrophils.

## MATERIALS and METHODS

C5a, phorbol 12-myristate 13-acetate (PMA), staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine dihydrochloride (H-7), genistein, methyl-2,5-dihydroxycinnamate, fura-2/AM, adenosine diphosphate (ADP) and thapsigargin were purchased from Sigma Chemical Co.. Other chemicals were of analytical grade.

### 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-Hypague density centrifugation (Markert *et al.*, 1984). The neutrophils were suspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.4 at a concentration of $1\times10^7$/ml. Final suspensions of neutrophils were comprised of about 97% neutrophils as judged by Wright-Giemsa stain, and viability was more than 98% as judged by trypan blue dye exclusion.

### Assay of cytosolic free calcium

Fura-2 loading and fluorescence measurement were performed by the method of Luscinskas *et al.* (1990). Neutrophils (approximately $5\times10^7$ cells/ml) were loaded with 2 mM fura-2/AM to 1 $\mu$M/$10^7$ cells at 37℃ for 10 min in the reaction mixtures contained Hanks' balanced salt solution (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 $5\times10^7$ cells/ml. Fluorescence measurement was done with a Turner Spectrofluorometer (Model 430). Preloaded neutrophils ($4\times10^6$) 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 C5a. The fluorescence change was read at an excitation wavelength of 340 nm and emission wavelength of 505 nm.

### 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 contained $4\times10^6$/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 protein kinase inhibitors at 37℃, the $Ca^{2+}$ release was initiated by adding C5a. 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 ($4\times10^6$/ml) were suspended in $Ca^{2+}$- and $Mg^{2+}$-containing HBSS media. After 1 min of stimulation with C5a, $Mn^{2+}$ (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.

### Assay of capacitative $Ca^{2+}$ entry

In thapsigargin (TG)-treated neutrophils, $Ca^{2+}$ entry was measured (Sargeant *et al.*, 1993). The reaction mixtures contained fura-2 loading neutrophils ($4\times10^6$ cell/ml), 200$\mu$M EGTA, 20 mM HEPES-tris and HBSS buffer without calcium, pH 7.4. After 5 min of preincubation with inhibitors, neutrophils were treated with 50 nM TG for 90 sec, and then 1 mM $Ca^{2+}$ was added to induce $Ca^{2+}$ influx.

## RESULTS

### Effects of protein kinase inhibitors on intracellular $Ca^{2+}$ mobilization

A 20 nM complement C5a caused an increase of 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 sec post addition, and then the $[Ca^{2+}]_i$ was gradually decreased to the resting level over the subsequent several minutes. The effect of protein kinase inhibition on C5a-induced $[Ca^{2+}]_i$ increase was studied. A preincubation of fura-2-loaded neutrophils with either 100 nM staurosporine (data not shown) or 50$\mu$M H-7, a protein kinase C inhibitor, did not affect the elevation of $[Ca^{2+}]_i$ evoked by 20 nM C5a (Fig. 1). However, protein tyrosine kinase inhibitors, 10$\mu$M genistein and 1$\mu$g/ml methyl-2,5-dihydroxycinnamate, had an inhibitory effect on the stimulatory action of C5a.

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. Addition of C5a induced the release of $Ca^{2+}$ from intracellular stores in fura-2-loaded neutrophils in $Ca^{2+}$ free media. In 1 mM EGTA containing media without external addition $Ca^{2+}$, 20 nM C5a caused an immediate elevation of $[Ca^{2+}]_i$ which is followed by sustained decrease of $[Ca^{2+}]_i$. Effects of protein kinase inhibitors on the intracellular $Ca^{2+}$ release

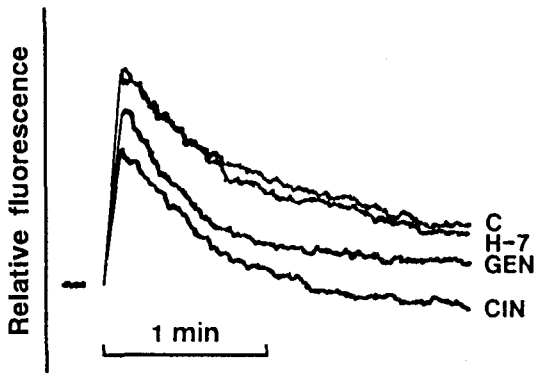


**Fig. 1.** Effects of protein kinase inhibitors on C5a-evoked elevation of $[Ca^{2+}]_i$. Fura-2 loaded neutrophils ($4\times10^6$ cells/ml) were preincubated with inhibitors or not (C) for 5 min, and then the response was initiated by the addition of 20 nM C5a. H-7, 50$\mu$M H-7; GEN, 10$\mu$M genistein; CIN, 1$\mu$g/ml methyl-2,5-dihydroxycinnamate. The traces are representative of three experiments.

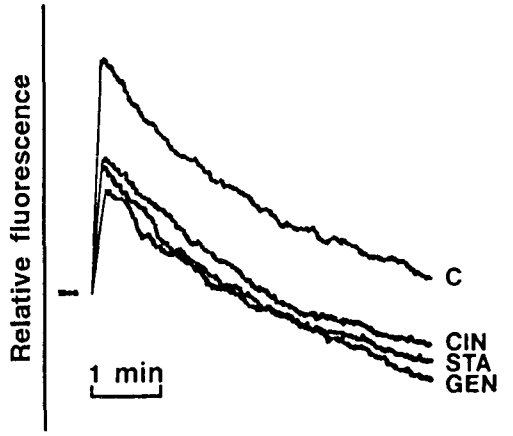


**Fig. 2.** Inhibitory effects of protein kinase inhibitors on the intracellular $Ca^{2+}$ release. In $Ca^{2+}$-free media, after preincubation of neutrophils with inhibitors or not (C) the intracellular $Ca^{2+}$ release was initiated by adding 20 nM C5a, STA, 100 nM staurosporine; GEN, 10$\mu$M genistein; CIN, 1$\mu$g/ml methyl-2,5-dihydroxycinnamate. The traces are representative of three experiments.

were examined. As shown in Fig. 2, the C5a-induced intracellular $Ca^{2+}$ release was inhibited by 100 nM staurosporine, 10$\mu$M genistein and 1 $\mu$g/ml methyl-2,5-dihydroxycinnamate.

$Mn^{2+}$ is considered to permeate through the neutrophil $Ca^{2+}$ influx pathway activated by chemoattractants (Merritt *et al.*, 1989; Demaurex *et al.*, 1992). The experiments were done at an excitation wavelength of 360 nm. In this wavelength, C5a did not cause fluorescence change in fura-2-loaded neutrophils. When added to 20 nM C5a-stimulated neutrophils, 0.5 mM $Mn^{2+}$ caused an immediate and continuous decrease in fluorescence. The involvements of protein kinase C and protein tyrosine kinase in $Ca^{2+}$ influx across the plasma membrane were investigated. Neutrophils were preincubated with protein kinase inhibitors for 5 min and then were exposed to 20 nM C5a for 1 min prior to $Mn^{2+}$ addition. A 100 nM staurosporine and 50 $\mu$M H-7 did not affect the $Mn^{2+}$ influx induced by C5a (Fig. 3). In contrast, the $Mn^{2+}$ influx induced by C5a was inhibited by 10 $\mu$M genistein and 1 $\mu$g/ml methyl-2,5-dihydroxycinnamate.

**Inhibition of ADP-evoked elevation of intracellular calcium by protein tyrosine kinase inhibitors**

Adenosine diphosphate (ADP) evokes a biphase elevation of $[Ca^{2+}]_i$ and induces protein-tyrosine phosphorylation in platelets (Sargeant *et al.*, 1993). In 1.23 mM $Ca^{2+}$ containing media, 10 $\mu$M ADP caused an immediate and sustained elevation of $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ was very slowly decreased. Influence of protein kinase inhibition on ADP-induced elevation of $[Ca^{2+}]_i$ was examined. Fig. 4 showes that a preincubation of neutrophils with either 100 nM staurosporine or 50 $\mu$M H-7 did not show any significant effect on ADP-induced elevation of $[Ca^{2+}]_i$, while 10 $\mu$M genistein and 1 $\mu$g/ml methyl-2,5-dihydroxycinnamate inhibited it.

**Inhibitory effects of protein tyrosine kinase inhibitors thapsigargin-induced $Ca^{2+}$ influx**

Thapsigargin (TG), an inhibitor of the endomembranous $Ca^{2+}$ ATPase, is thought to deplete the intracellular $Ca^{2+}$ stores without in-

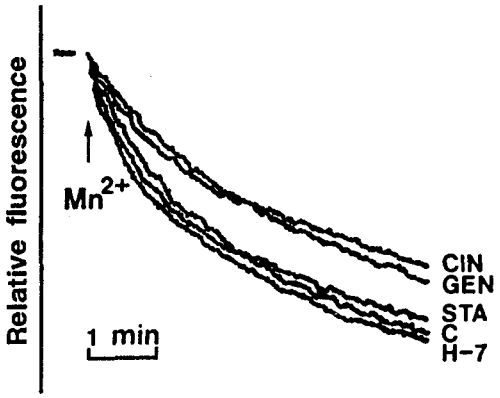


Fig. 3. Effects of protein kinase inhibitors on $Mn^{2+}$ influx. $Mn^{2+}$ influx into the cytoplasm of neutrophil was initiated by adding 0.5 mM $Mn^{2+}$ after 1 min of stimulation with 20 nM C5a. Fura-2 loaded neutrophils ($4\times10^6$ cells/ml) were preincubated with inhibitors or not (C) for 5 min, and then the response was initiated by addition of 20 nM C5a, STA, 100 nM staurosporine; H-7, 50 $\mu$M H-7; GEN, 10 $\mu$M genistein; CIN, 1 $\mu$g / ml methyl-2,5-dihydroxycinnamate. The traces are representative of three experiments.

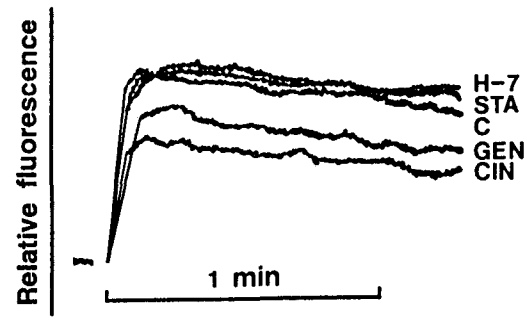


Fig. 4. Inhibition of ADP-evoked elevation of $[Ca^{2+}]_i$ by protein tyrosine kinase inhibitors. Fura-2 loaded neutrophils ($4\times10^6$ cells/ml) were preincubated with inhibitors or not (C) for 5 min, and then the response was initiated by the addition of 10 $\mu$M ADP. STA, 100 nM staurosporine; H-7, 50 $\mu$M H-7; GEN, 10 $\mu$M genistein; CIN, 1 $\mu$g/ml methyl-2,5-dihydroxycinnamate. The traces are representative of three experiments.

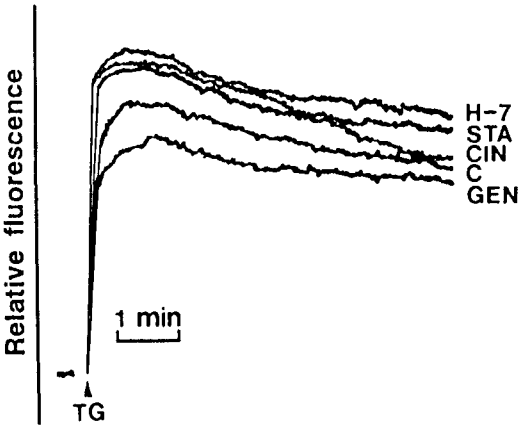


Fig. 5. Inhibitory effects of protein tyrosine kinase inhibitors on thapsigargin-induced $Ca^{2+}$ influx. In $Ca^{2+}$ media, fura-2 loaded neutrophils were preincubation with inhibitors or not (C), and then 50 nM thapsigargin (TG) was added. At 90 sec of post addition of TG, 1 mM $CA^{2+}$ was added to induce $Ca^{2+}$ influx. STA, 100 nM staurosporine; H-7, 50 $\mu$M H-7; GEN, 10 $\mu$M genistein; CIN, 1 $\mu$g/ml methyl-2,5-dihydroxycinnamate. The traces are representative of three experiments.

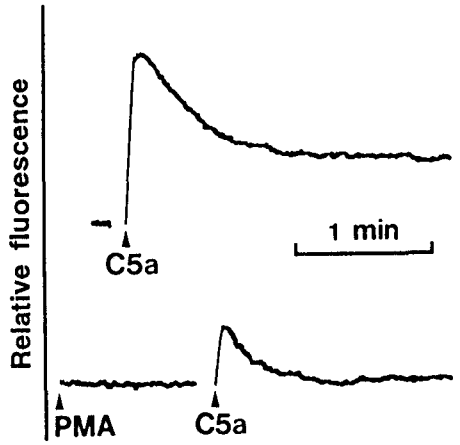


Fig. 6. Effects of protein kinase C activation on C5a-induced elevation of $[Ca^{2+}]_i$. Fura-2 loaded neutrophils were incubated with 0.1 $\mu$g/ml PMA for 90 sec, and then 20 nM C5a was added. The traces are representative of three experiments.

creasing cellular InsP3 (Jackson *et al.*, 1988). The depletion of intracellular $Ca^{2+}$ pools appears to induce $Ca^{2+}$ entry across the plasma membrane (Demaurex *et al.*, 1992; Putney, 1993). In $Ca^{2+}$ free media, fura-2-loaded neutrophils were treated with TG for 90 sec to deplete intracellular $Ca^{2+}$ stores and then were exposed to high concentration of $Ca^{2+}$. TG itself did not cause any recognable change of fluorescence for the stated time. As shown in Fig. 5, the addition of 1 mM $Ca^{2+}$ to TG-treated neutrophils evoked a marked elevation of $[Ca^{2+}]_i$. Effects of protein kinase inhibitors on this capacitative $Ca^{2+}$ entry were studied. Preincubation with either 100 nM staurosporine or 50 $\mu$M H-7 did not have an inhibitory effect on TG-induced $Ca^{2+}$ influx. However, in the presence of 10 $\mu$M genistein or 1 $\mu$g/ml methyl-2,5-dihydroxycinnamate, TG-induced $Ca^{2+}$ influx was significantly decreased.

**Effect of protein kinase C activation C5a-induced elevation of intracellular calcium**

It has been suggested that activation of protein kinase C with PMA decrease fMLP-induced elevation of $[Ca^{2+}]_i$ by blocking of $Ca^{2+}$ influx from the extracellular medium and by partially inhibition of intracellular $Ca^{2+}$ release (McCarthy *et al.*, 1989). In 1.23 mM $Ca^{2+}$ containing media, neutrophils were preactivated by addition of 0.1 $\mu$g/ml PMA for 90 sec, and then 20 nM C5a added. PMA itself did not cause any significant fluorescence change. When preincubation with PMA, the eliciting action of C5a on $[Ca^{2+}]_i$ was markedly inhibited, and a slight increase of $[Ca^{2+}]_i$ occured (Fig. 6).

## DISCUSSION

Changes in cytosolic free calcium concentration appear to play a central role in the activation process, including translocation of protein kinase C. Decrease of intracellular calcium, either by intracellular calcium antago-

nists (Smolen *et al.*, 1981) or by addition of $Ca^{2+}$ chelators such as EGTA (Campbell and Hallett, 1983), reduces aggregation, lysosomal enzyme release and superoxide production in response to various stimulating agents. Elevation of $[Ca^{2+}]_i$ is an early event in the response of neutrophils to agonists, including fMLP, C5a and platelet-activating factor (Westwick and Poll, 1986). The binding of chemoattractants to receptors on the plasma membrane elicites a biphasic increase in $[Ca^{2+}]_i$. A rapid and transient initial phase is attributed to release from the intracellular $Ca^{2+}$ stores, and a sustained phase, which is maintained by influx from the extracellular medium, is followed (Westwick and Poll, 1986; Cobbold and Rink, 1987).

Complement C5a caused an immediate increase of $[Ca^{2+}]_i$ in fura-2-loaded neutrophils. The elevated $[Ca^{2+}]_i$ was gradually decreased. Receptor-mediated intracellular calcium mobilization is though to be coupled to phospholipase C activation which promotes phosphoinositide hydrolysis with the formation of $InsP_3$ and DAG (Nishizuka, 1984; Berridge, 1987). The initial intracellular $Ca^{2+}$ release is mediated by $InsP_3$. However, the mechanism underlying receptor-mediated $Ca^{2+}$ influx is uncertain. $InsP_4$ may be responsible for the activation of $Ca^{2+}$ influx (Lückhoff and Clapham, 1992). In addition, it has been suggested that the decrease in the $Ca^{2+}$ content of intracellular pool activates a pathway for entry into the pool from the extracellular medium (Demaurex *et al.*, 1992). Activation of C5a receptors evoked both intracellular $Ca^{2+}$ release and $Ca^{2+}$ entry across the plasma membrane. Thus, this finding supports that after receptor binding the intracellular $Ca^{2+}$ mobilization is attained by both intracellular $Ca^{2+}$ release and $Ca^{2+}$ influx.

The involvements of protein kinase C and protein tyrosine kinase in the intracellular $Ca^{2+}$ mobilization in C5a-stimulated neutrophils were studied. Staurosporine and H-7 had a different effect on the $Ca^{2+}$ release and influx. They inhibited intracellular $Ca^{2+}$ release but did not affect $Ca^{2+}$ influx. Thus, intracellular $Ca^{2+}$ release in C5a- stimulated neutrophils may be regulated by protein kinase C, while protein kinase C probably does not affect $Ca^{2+}$ influx. Meanwhile, both phase in the elevation of $[Ca^{2+}]$ induced by C5a was inhibited by genistein and methyl-2,5-dihydroxycinnamate. In C5a-stimulated neutrophils, protein tyrosine kinase appears to be involved partly in the elevation of $[Ca^{2+}]_i$ by its regulatory action on intracellular $Ca^{2+}$ release and $Ca^{2+}$ influx across the plasma membrane. Alteration of intracellular $Ca^{2+}$ release alone by protein kinase C may not attribute the elevation of $[Ca^{2+}]_i$.

ADP induces protein-tyrosine phosphorylation and the elevation of $[Ca^{2+}]$ in platelets, which is inhibited by protein tyrosine kinase inhibitors, genistein and methyl-2,5-dihydroxycinnamate (Sage *et al.*, 1989; Sargeant *et al.*, 1993). The inhibitory effects of protein kinase inhibitors on ADP-evoked elevation of $[Ca^{2+}]_i$ were also observed in this study. Thus, the participation of protein tyrosine kinase in the intracellular $Ca^{2+}$ mobilization is suggested. However, it is unlikely that protein kinase C may be involved in the elevation of $[Ca^{2+}]_i$ induced by ADP.

Thapsigargin (TG) is known to deplete the intracellular $Ca^{2+}$ stores without inositol phosphate formation. It is postulated that TG-induced depletion of intracellular pools appears to enhance tyrosine phosphorylation and promote $Ca^{2+}$ entry across the plasma membrane (Vostal *et al.*, 1991; Sargeant *et al.*, 1993). The addition of 1 mM $Ca^{2+}$ to TG-treated neutrophils in $Ca^{2+}$ free media caused a marked elevation of $[Ca^{2+}]_i$. The inhibitory effects of genistein and methyl-2,5-dihydroxycinnamate indicate that protein tyrosine kinase may control $Ca^{2+}$ entry in neutrophils. Meanwhile, the implication of protein kinase C in the store-regulated $Ca^{2+}$ entry is not suggested. These results suggest that protein tyrosine kinase play an important regulatory role in intracellular $Ca^{2+}$ mobilization in neutrophils rather than protein kinase C. It has been reported that activation of protein kinase C inhibits agonist-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils by blocking influx of bivalent cations from the extracellular medium and by stimulating $Ca^{2+}$ efflux (McCarthy *et al.*, 1989). In addition, protein kinase C activation leads to decreased InsP3 formation and therefore decreased intracellular $Ca^{2+}$ release (Della Bianca *et al.*, 1986). The present finding is coincided with previous data. Com-

plement C5a-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils could be attenuated by protein kinase C activation.

## REFERENCES


Becker EL, Kermode JC, Naccache PH, Yassin R, Marsh ML, Munoz JJ and Sha'afi RI: *The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. J Cell Biol 100: 1640-1646, 1985*

Berkow RL and Dodson RW: *Tyrosine-specific protein phosphorylation during activation of human neutrophils. Blood 75: 2445-2452, 1990*

Berridge MJ: *Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 56: 159-193, 1987*

Campbell AK and Hallett MB: *Measurement of intracellular calcium ions and oxygen radicals in polymorphonuclear leucocyte erythrocyte ghost hybrids. J Physiol 338: 537-550, 1983*

Cobbold PH and Rink TJ: *Fluorescence and bioluminescence measurement of cytoplasmic free calcium. Biochem J 248: 313-328, 1987*

Della Bianca V, Grzeskowiak M, Cassatella M, Zeni L and Rossi F: *Phorbol myristate acetate potentiates the respiratory burst while it inhibits phosphoinositide hydrolysis and calcium mobilization by formyl-methionyl-leucyl-phenylalanine in human neutrophils. Biochem Biophys Res Commun 135: 556-565, 1986*

Demaurex N, Lew DP and Krause KH: *Cyclopiazonic acid depletes intracellular $Ca^{2+}$ stores and activates an influx pathway for divalent cations in HL-60 cells. J Biol Chem 267: 2318-2324, 1992*

Dougherty RW, Godfrey PP, Hoyle PC, Putney JWJr and Freer RJ: *Secretagogue-induced phosphoinositide metabolism in human leukocytes. Biochem J 222: 307-314, 1984*

Fantone JC and Ward PA: *Role of oxygen derived free radicals and metabolites in leukocyte-dependent inflammatory reaction. Am J Pathol 107: 397-418, 1982*

Gil MG, Alonso VA, Crespo MS and Mato JM: *Phospholipid turnover during phagocytosis in human polymorphonuclear leukocytes. Biochem J 206: 67-72, 1982*

Goldstein IM: *Complement. Biologically active products. Inflammation: Basic principles and clinical correlates. Second edition (Gallin JI, Goldstein IM and Synderman R, ed.) Raven Press, Ltd., New York, 1992*

Irvine RF and Moor RM: *Micro-injection of inositol 1, 3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external $Ca^{2+}$. Biochem J 240: 917-920, 1986*

Jackson TR, Patterson SI, Thastrup O and Hanley MR: *A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free $Ca^{2+}$ without generation of inositol phosphates in NG115-401L neuronal cells. Biochem J 253: 81-86, 1988*

Krause KH, Schlegel W, Wollheim CB, Andersson T, Waldvogel FA and Lew DP: *Chemotactic peptide activation of human neutrophils and HL-60-cells. J Clin Invest 76: 1348-1354, 1985*

L ckhoff A and Clapham DE: *Inositol 1,3,4,5-tetrakisphosphate activates an endothelial $Ca^{2+}$-permeable channel. Nature 355: 356-358, 1992*

Luscinskas FW, Nicolaou KC, Webber SE, Veale CA, Gimbrone MAJr and Serhan CN: *$Ca^{2+}$ mobilization with leukotriene A4 and epoxytetraenes in human neutrophils. Biochem Pharmacol 39: 355-365, 1990*

Markert M, Andrews PC and Babior BM: *Measurement of $O_2^-$ production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. Methods Enzymol (Packer L, ed.) 105: pp 358-365. Academic Press Inc, 1984*

McCarthy SA, Hallam TJ and Merritt JE: *Activation of protein kinase C in human neutrophils attenuates agonist-stimulated rises in cytosolic free $Ca^{2+}$ concentration by inhibiting bivalent-cation influx and intracellular $Ca^{2+}$ release in addition to stimulating $Ca^{2+}$ efflux. Biochem J 264: 357-364, 1989*

Merritt JE, Jacob R and Hallam TJ: *Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. J Biol Chem 264: 1522-1527, 1989*

Nishizuka Y: *The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308: 693-698, 1984*

*Parys JB, Missiaen L, De Smedt H and Casteels R: Loading dependence of inositol 1,4,5-trisphosphate-induced $Ca^{2+}$ release in the clonal cell line A7r5. Implications for the mechanism of quantal $Ca^{2+}$ release. J Biol Chem 268: 25206-25212, 1993*

Pozzan T, Lew DP, Wollheim CB and Tsien RY: *Is cytosolic ionised calcium regulating neutrophil activation. Science 221: 1413-1415, 1983*

Putney JWJr: *A model for receptor-regulated calcium entry. Cell Calcium 7: 1-12, 1986*

Putney JWJr: *Receptor-regulated calcium entry. Intracellular messengers. (Taylor CW, ed.) pp 255-263. Pergamon Press Ltd., 1993*

Sage SO, Merritt JE, Hallam TJ and Rink TJ: *Recep-*

*tor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin: dual wavelengths studies with $Mn^{2+}$. Biochem J 258: 923-926, 1989*

Sargeant P, Farndale RW and Sage SO: *ADP-and thapsigargin-evoked $Ca^{2+}$ entry and protein-tyrosine phosphorylation are inhibited by the tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate in fura-2-loaded human platelets. J Biol Chem 268: 18151-18156, 1993*

Smolen JE, Korchak HM and Weissmann G: *The roles of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human neutrophils. Biochim Biophys Acta 677: 512-520, 1981*

Vostal JG, Jackson WL and Shulman NR: *Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. J Bol Chem 266: 16911-16916, 1991*

Westwick J and Poll C: *Mechanisms of calcium homeostasis in the polymorphonuclear leukocyte. Agents and Actions 19: 80-86, 1986*

=국문초록=

# C5a에 의해 자극된 호중구에서 세포내 칼슘동원에 대한 Protein Tyrosine Kinase의 조절작용

중앙대학교 의과대학 약리학교실

**최 원 태 · 한 은 숙 · 이 정 수***

본 연구는 C5a에 의해 자극된 호중구에서 세포내 칼슘유리와 세포외부로부터 칼슘유입에 있어 protein kinase C와 protein tyrosine kinase의 관여 여부를 조사하였다.

Protein kinase C 억제제인 staurosporine과 H-7은 C5a에 의해 자극된 호중구에서 세포내 칼슘유리를 억제하였으나, 세포막을 교차한 칼슘유입이나 세포내 칼슘농도 증가에 영향을 나타내지 않았다. C5a에 의한 세포내 칼슘유리와 칼슘유입은 protein tyrosine kinase 억제제인 genistein과 methyl-2,5-dihydroxycinnamate에 의해서 억제 되었다. ADP에 의해 야기된 세포내 칼슘농도의 증가는 genistein과 methyl-2,5-dihydroxycinnamate에 의해서 억제되었으나 staurosporine과 H-7의 영향은 받지 않았다. Genistein과 methyl-2,5-dihydroxy-cinnamate는 thapsigargin을 처리한 호중구에서 칼슘유입을 감소시켰으나 이에 대한 staurosporine과 H-7의 효과는 나타나지 않았다. 호중구를 phorbol 12-myristate 13-acetate로 전처치하였을때 세포내 칼슘증가에 미치는 C5a의 자극 효과는 감소하였다.

이상의 결과로 부터 protein tyrosine kinase는 C5a에 의해 활성화된 호중구에서 세포내 칼슘유리와 세포막을 교차한 칼슘유입의 조절에 관여할 것으로 추정된다.