

Depression of Ca^{2+} Influx in Complement C5a-stimulated Neutrophils by Calmodulin Inhibitors

Dong Suk Ham, Hyun Ho Kim, Eun Sook Han, and Chung Soo Lee

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

Role of Ca^{2+} /calmodulin complex in intracellular Ca^{2+} mobilization in neutrophils has not been clearly elucidated. In this study, effects of chlorpromazine, trifluoperazine and imipramine on the intracellular Ca^{2+} mobilization, including Ca^{2+} influx, in C5a-activated neutrophils were investigated. Complement C5a-stimulated superoxide production and myeloperoxidase release in neutrophils were inhibited by chlorpromazine, trifluoperazine and imipramine, except no effect of imipramine on myeloperoxidase release. A C5a-elicited elevation of $[\text{Ca}^{2+}]_i$ in neutrophils was inhibited by chlorpromazine, trifluoperazine, imipramine, staurosporine, genistein, EGTA, and verapamil but not affected by pertussis toxin. The intracellular Ca^{2+} release in C5a-activated neutrophils was not affected by chlorpromazine and imipramine. Chlorpromazine and imipramine inhibited Mn^{2+} influx by C5a-activated neutrophils. Thapsigargin-evoked Ca^{2+} entry was inhibited by chlorpromazine, trifluoperazine, imipramine, genistein, EGTA and verapamil, while the effect of staurosporine was not detected. The results suggest that Ca^{2+} /calmodulin complex is involved in the activation process of neutrophils. The depressive action of calmodulin inhibitors on the elevation of cytosolic Ca^{2+} level in C5a-activated neutrophils appears to be accomplished by inhibition of Ca^{2+} influx from the extracellular medium.

Key Words: Calmodulin inhibitors, Intracellular Ca^{2+} mobilization, Complement C5a, Neutrophils

INTRODUCTION

Complement C5a is a principal mediator of inflammatory responses (Goldstein, 1992). C5a is a potent chemotaxin for neutrophils and macrophages and stimulates these cells to release lysosomal enzymes and to produce superoxide anion (Goldstein et al, 1975; Goldstein, 1992). The effects of C5a are amplified by chemical mediators. C5a binds to surface receptor which is associated with pertussis toxin-sensitive guanine nucleotide-binding proteins (G proteins), and produces its biological effects (Becker et al, 1985).

Change in the level of free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) accompany neutrophil activation and has been considered to play a significant role in the early

triggering of activation of neutrophil response (Smolen et al, 1981; Painter et al, 1984). Binding of chemoattractants to receptors elicits an elevation of $[\text{Ca}^{2+}]_i$. The respiratory burst induced by chemoattractants can be inhibited by Ca^{2+} chelators, such as EGTA and a calmodulin antagonist, trifluoperazine (Smolen et al, 1981; Campbell & Hallett, 1983).

Chlorpromazine, a calmodulin inhibitor, inhibits both opsonized zymosan-or phorbol 12-myristate 13-acetate (PMA)-activated superoxide production and platelet-activating factor-induced degranulation in phagocytic cells (Cohen et al, 1980; Lee et al, 1993). The A23187-or fMet-Leu-Phe (fMLP)-induced cAMP elevation in neutrophils is inhibited by calmodulin inhibitors, chlorpromazine and trifluoperazine (Iannone et al, 1991). In addition to effects on calmodulin, they have been shown to inhibit phospholipid sensitive calcium-dependent protein kinases (Schatzman et al, 1981; Wise et al,

Corresponding to: Chung Soo Lee, Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

1982). Imipramine appears to have an inhibitory action on calcium-calmodulin complex (Barritt, 1992). PMA is thought to stimulate superoxide production in neutrophils without change of intracellular Ca^{2+} level (Tauber, 1987). The inhibitory action mechanisms of chlorpromazine and imipramine on the responses of activated neutrophils have not been clearly elucidated. Both calmodulin at low Ca^{2+} mixture conditions (Ikemoto et al, 1995) and trifluoperazine (Ikemoto et al, 1996) potentiate Ca^{2+} -induced Ca^{2+} release from the sarcoplasmic reticulum. The role of calcium-calmodulin complex in intracellular calcium mobilization in activated neutrophils is also uncertain.

In this study, effects of chlorpromazine, trifluoperazine, and imipramine on respiratory burst, degranulation, intracellular Ca^{2+} mobilization in C5a-activated neutrophils were investigated.

METHODS

Complement C5a, chlorpromazine, trifluoperazine, imipramine, staurosporine, genistein, pertussis toxin, ethyleneglycol-bis (β -amino-ethylether), N, N, N', N', tetraacetic acid (EGTA), verapamil, ferricytochrome c, o-dianisidine HCl, fura-2/AM, thapsigargin, cytochalasin B, and Hanks' balanced salt solution (HBSS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of analytical reagent 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-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 cells/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 superoxide production

The superoxide dependent reduction of ferri-

cytochrome c was measured by the method of Markert et al. (1984). The reaction mixtures in plastic microfuge tubes contained 2×10^6 neutrophils, $75 \mu\text{M}$ ferricytochrome c, C5a, 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 5 min. The reaction was then stopped by placing the tubes in melting ice, and the cells were rapidly pelleted by centrifuging at 800 g for 5 min at 4°C . The supernatants were taken, and the amount of reduced cytochrome c was calculated by using an extinction coefficient of $2.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 550 nm (Cohen & Chovaniec, 1978).

After neutrophils were pretreated with cytochalasin B ($5 \mu\text{g/ml}$ for 10^7 cells) for 5 min, the assay for the respiratory burst and degranulation was done.

Assay of hydrogen peroxide production

H_2O_2 produced from activated neutrophils was measured by a change of scopoletin fluorescence. The reaction mixtures contained 2×10^6 neutrophils, $2.5 \mu\text{M}$ scopoletin, $5 \mu\text{g/ml}$ horseradish peroxidase, C5a and HBSS buffer in a total volume of 1.0 ml. After preincubation of 5 min at 37°C with compounds, the reaction was initiated by the addition of stimulating agents. The decrease of scopoletin fluorescence by H_2O_2 produced was read at the wavelengths of excitation, 343 nm and emission, 460 nm (Root et al, 1975).

Assay of myeloperoxidase release

Measurement of myeloperoxidase release was done by the method of Spangrude et al. (1985). A 5×10^6 cells/ml neutrophils in HBSS buffer with or without inhibitors were stimulated by adding 20 nM C5a at 37°C . 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. Myeloperoxidase activity was determined by the change in absorbance at 450 nm (ΔA_{450}) using the equation, dianisidine oxidation (n mol) = $50 \times \Delta A_{450}$ (Burt et al, 1994).

Assay of cytosolic free calcium

Fura-2 loading, fluorescence measurement, and assay of Ca^{2+} amount were performed by the method of Lusinskas et al. (1990). Neutrophils (approximately 5×10^7 cells/ml) were loaded with 2 mM fura-2/AM ($1 \mu\text{M}/10^7$ 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 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 measurements were made using a Turner Spectrofluorometer (Model 430). Preloaded neutrophils (4×10^6) were suspended in 1.23 mM Ca^{2+} and 1 mM Mg^{2+} containing HBSS 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 C5a. The fluorescence change was read at an excitation wavelength of 340 nm and an emission wavelength of 505 nm. The traces on intracellular Ca^{2+} mobilization are 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 contained neutrophils (4×10^6 /ml, fura-2 loaded), 1 mM EGTA, 1 mM MgCl_2 , HBSS, and 20 mM HEPES-tris, pH 7.4 without extracellular added Ca^{2+} . After 5 min of preincubation with or without inhibitors at 37°C , 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 (De-maurex et al, 1992). Fura-2 loaded neutrophils (4×10^6 /ml) were suspended in Ca^{2+} - and Mg^{2+} -

containing HBSS media. After 2 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-treated neutrophils, Ca^{2+} entry was measured (Sargeant et al, 1993). The reaction mixtures contained fura-2 loading neutrophils (4×10^6 cell/ml), 200 μM 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 thapsigargin for 90 sec, and then 1 mM Ca^{2+} was added to induce Ca^{2+} influx.

Data analysis

The results in experiments of respiratory burst and myeloperoxide release were analyzed for level of significance using the Student's t-test.

RESULTS

Inhibition of C5a-induced respiratory burst and degranulation by calmodulin inhibitors

Complement C5a activated neutrophils to stimulate functional responses. C5a stimulated superoxide production by neutrophils, and at 20 nM $16.75 \pm 1.28 \text{ nmol of superoxide anion}/5 \text{ min}/2 \times 10^6 \text{ cells}$ ($n=6$) was produced. Role of Ca^{2+} -calmodulin complex in the activation process of respiratory burst was studied. As shown in Fig. 1, a 50 μM to 100 μM of calmodulin inhibitors, chlorpromazine, trifluoperazine and imipramine inhibited 20 nM C5a-induced superoxide production by 78.8%, 61.9% and 50.5%, respectively. A C5-induced superoxide production was inhibited by 100 nM staurosporine and 10 μM genistein.

PMA selectively stimulates protein kinase C without activation of G proteins to cause neutrophil responses (Tauber, 1987). Influences of calmodulin inhibitors on activation of protein kinase C were observed. The stimulatory action of 0.1 $\mu\text{g/ml}$ PMA on superoxide production was inhibited by 50 μM chlorpromazine, 100 μM im-

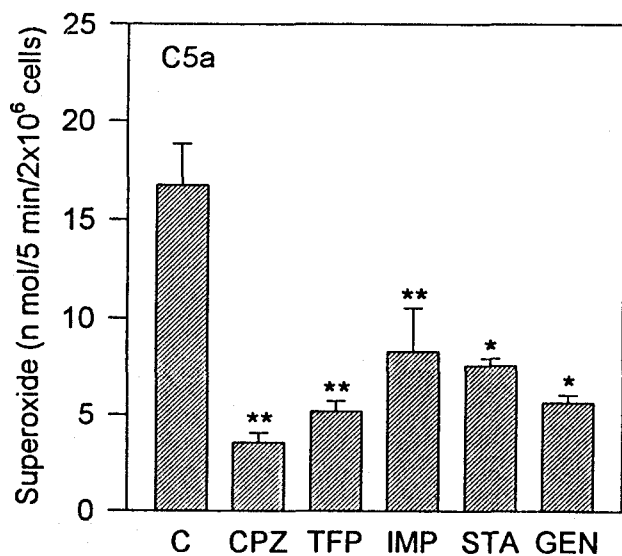


Fig. 1. Inhibition of C5a-induced superoxide production by chlorpromazine and imipramine. Neutrophils were stimulated with 20 nM C5a in the presence of 50 μ M chlorpromazine (CPZ), 50 μ M trifluoperazine (TFP), 100 μ M imipramine (IMP), 100 nM staurosporine (STA) and 10 μ M genistein (GEN) or without reagent (C). Values are means \pm SD, n=6. * p < 0.05, ** p < 0.01 by Student's t-test.

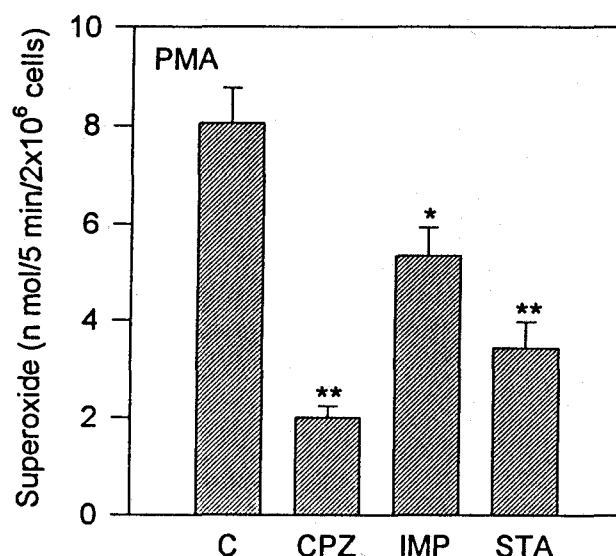


Fig. 2. Inhibitory effects of chlorpromazine and imipramine on PMA-stimulated with 0.1 μ g/ml PMA in the presence of 50 μ M chlorpromazine (CPZ), 100 μ M imipramine (IMP) and 100 nM staurosporine (STA) or without reagent (C), respectively. Values are means \pm SD, n=3. * p < 0.05, ** p < 0.01 by Student's t-test.

ipramine and 100 nM staurosporine (Fig. 2).

Hydrogen peroxide production, which is attained from the dismutation of superoxide anion (Fridovich, 1975), was measured by change of scopoletin fluorescence. Oxidation of scopoletin by neutrophils was stimulated by the addition of C5a. As shown in Table 1, H₂O₂ production in neutrophils activated by 20 nM C5a was almost completely inhibited by 50 μ M chlorpromazine, 50 μ M trifluoperazine and 100 μ M imipramine. Thus, the participation of calmodulin in production process of H₂O₂ is suggested.

The secretion of lysosomal enzymes from neutrophils was assayed by measuring the release of myeloperoxidase. The involvement of calmodulin in the process of degranulation was studied. As can be seen in Fig. 3, 50 μ M chlorpromazine, 50 μ M trifluoperazine and 10 μ M genistein inhibited myeloperoxidase release from neutrophils activated by 20 nM C5a, while the effect of 100 μ M imipramine and 100 nM staurosporine were not detected.

Table 1. Inhibitory effects of calmodulin inhibitors on C5a-induced hydrogen peroxide production

Compounds	Arbitrary units of fluorescence
20 nM C5a	26.5 \pm 2.0
+ 50 μ M Chlorpromazine	0.7 \pm 0.3**
+ 50 μ M Trifluoperazine	1.8 \pm 0.3**
+ 100 μ M Imipramine	3.7 \pm 0.4**

After 5 min of preincubation with inhibitors, neutrophils were stimulated with 20 nM C5a. Values are means \pm SD, n=3-6. ** p < 0.01 by Student's t-test.

Alteration of intracellular Ca²⁺ mobilization by calmodulin inhibitors

The cytosolic Ca²⁺ level was assayed by measuring fluorescence change of fura-2 due to the formation of fura-2 and Ca²⁺ complex. A 20 nM C5a elicited an instantaneous elevation of [Ca²⁺]_i after the addition. Influence of calmodulin inhibition on intracellular Ca²⁺ mobilization was investigated. As shown in Fig. 4, C5a-induced eleva-

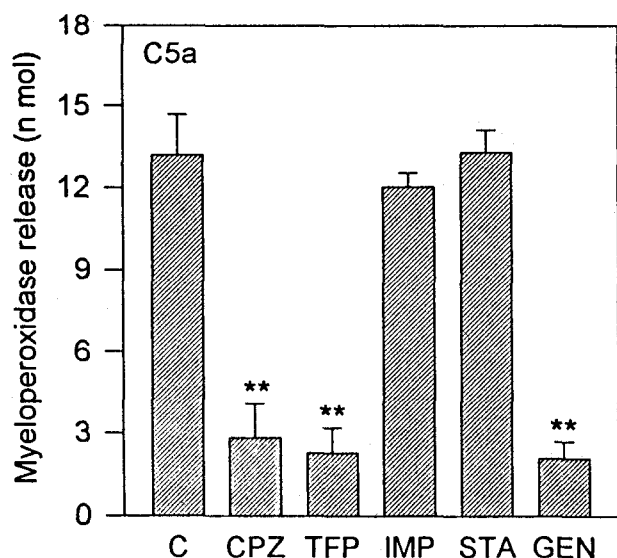


Fig. 3. Effects of calmodulin inhibitors on myeloperoxidase release. After 5 min of preincubation with 50 μM chlorpromazine (CPZ), 50 μM trifluoperazine (TFP), 100 μM imipramine (IMP), 100 nM staurosporine (STA) and 10 μM genistein (GEN) or without reagent (C), neutrophils were stimulated with 20 nM C5a. Values are means \pm SD, $n=4$. ** $p < 0.01$ by Student's *t*-test.

tion of $[\text{Ca}^{2+}]_i$ was inhibited by 50 μM chlorpromazine, 50 μM trifluoperazine and 100 μM imipramine. In this reaction, chlorpromazine showed an almost complete inhibition. Role of protein kinases in intracellular Ca^{2+} mobilization induced by C5a was examined. Fig. 4 shows that 100 nM staurosporine, 10 μM genistein, 5 mM EGTA and 100 μM verapamil inhibited elevation of $[\text{Ca}^{2+}]_i$ in neutrophils by C5a, whereas 0.1 $\mu\text{g/ml}$ pertussis toxin did not affect it.

The elevation of $[\text{Ca}^{2+}]_i$ 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 intracellular stores was induced by adding C5a. Effect of calmodulin inhibition on intracellular Ca^{2+} release was examined. As shown in Fig. 5, a 50 μM chlorpromazine and 100 μM imipramine did not affect intracellular Ca^{2+} release in neutrophils stimulated by 20 nM C5a.

The activity of the Ca^{2+} influx pathway was assayed with Mn^{2+} influx. The Mn^{2+} is thought to permeate through the neutrophil Ca^{2+} influx pathway activated by chemoattractants (Demaurex

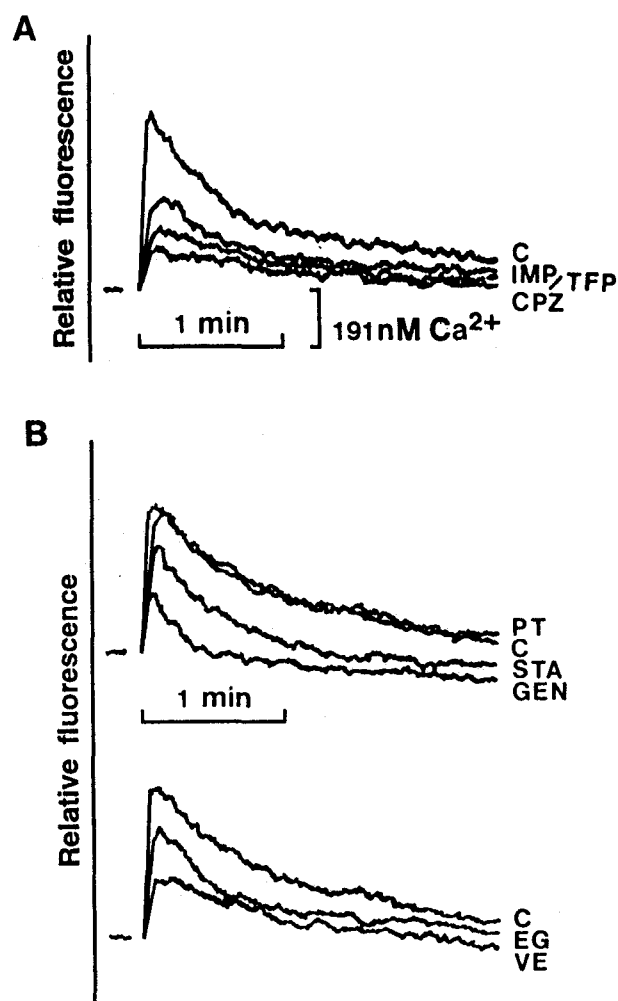


Fig. 4. Inhibition of C5a-induced elevation of $[\text{Ca}^{2+}]_i$ by calmodulin inhibitors. Fura-2 loaded neutrophils (4×10^6 cells/ml) were stimulated with 20 nM C5a in the presence of 50 μM chlorpromazine (CPZ), 50 μM trifluoperazine (TFP), 100 μM imipramine (IMP) (A); 100 nM staurosporine (STA), 10 μM genistein (GEN), 0.1 $\mu\text{g/ml}$ pertussis toxin (PT), 5 mM EGTA (EG), and 100 μM verapamil (VE) (B) or absence of reagent (C).

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. At 90 sec after stimulation of neutrophils with 20 nM C5a, Mn^{2+} influx through the Ca^{2+} influx pathway was initiated by adding 0.5 mM Mn^{2+} . The stimulated increase in Mn^{2+} influx was inhibited by 50 μM of chlorpromazine and 100 μM imipramine (Fig. 6).

Regulatory action of calmodulin on the Ca^{2+}

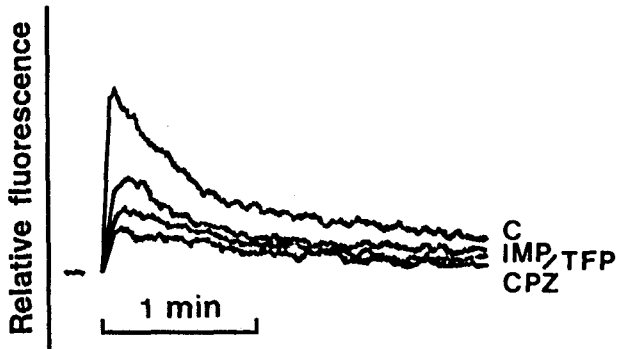


Fig. 5. Effects of chlorpromazine and imipramine on the intracellular Ca^{2+} release. In Ca^{2+} free media, after preincubation of neutrophils with $50 \mu\text{M}$ of chlorpromazine (CPZ) and $100 \mu\text{M}$ imipramine (IMP) or without reagent (C), the assay was done.

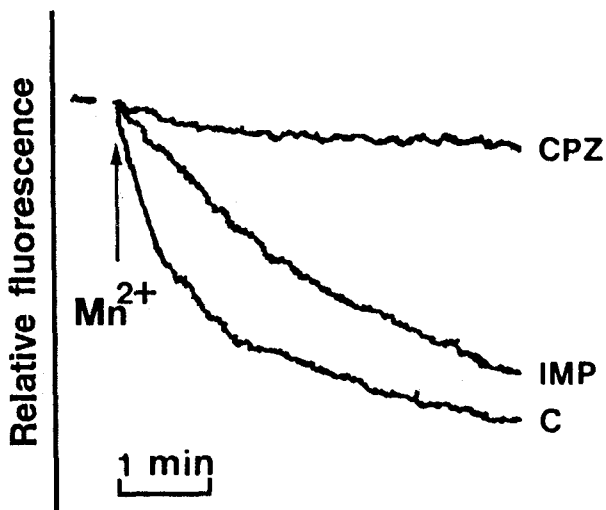


Fig. 6. Effects of chlorpromazine and imipramine on Mn^{2+} influx induced by C5a. Neutrophils were preincubated with either $50 \mu\text{M}$ of chlorpromazine (CPZ) and $100 \mu\text{M}$ imipramine (IMP) or without reagent (C). After this, assay of Mn^{2+} influx was done.

influx across the plasma membrane was also assayed with thapsigargin-treated neutrophils. Thapsigargin, which inhibits intracellular Ca^{2+} - Mg^{2+} ATPase, depletes the intracellular medium. Fig. 7 shows that the addition of 1mM Ca^{2+} to 50nM thapsigargin-pretreated neutrophils resulted in Ca^{2+} influx and then elicited elevation of $[\text{Ca}^{2+}]_i$. In 50nM thapsigargin-treated cells, preincubation of $50 \mu\text{M}$ chlorpromazine, $50 \mu\text{M}$ trifluoperazine and $100 \mu\text{M}$ imipramine inhibited the Ca^{2+} entry

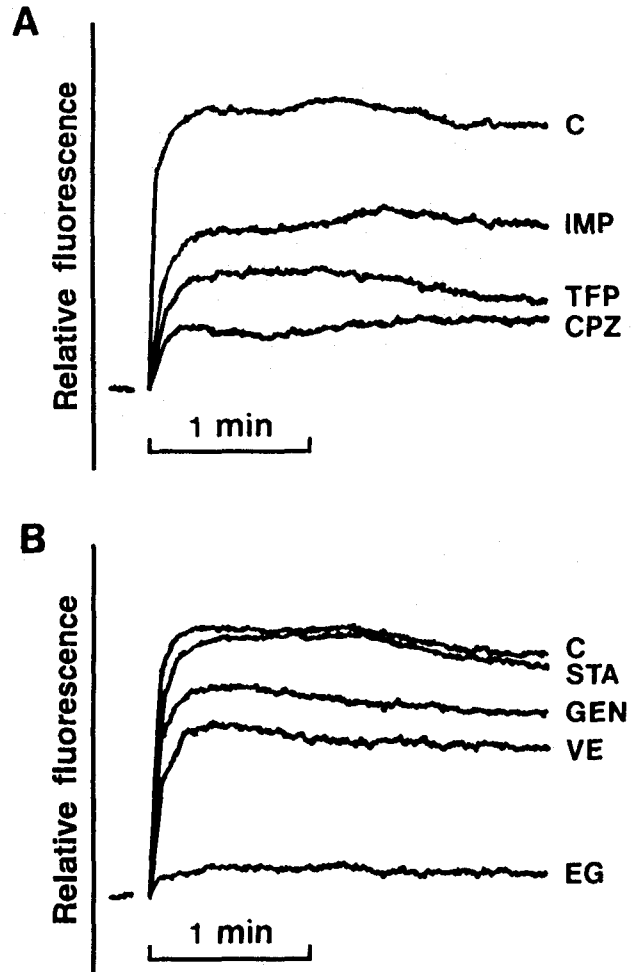


Fig. 7. Effects of calmodulin inhibitors on the elevation of $[\text{Ca}^{2+}]_i$ evoked by thapsigargin. Neutrophils were preincubated with either $50 \mu\text{M}$ chlorpromazine (CPZ), $50 \mu\text{M}$ trifluoperazine (TFP), and $100 \mu\text{M}$ imipramine (IMP) (A); 100nM staurosporine (STA), $10 \mu\text{M}$ genistein (GEN), 5mM EGTA (EG), and $100 \mu\text{M}$ verapamil (VE) (B) or without reagent (C).

induced by subsequently adding 1mM Ca^{2+} . The Ca^{2+} entry in thapsigargin-pretreated neutrophils were inhibited by $10 \mu\text{M}$ genistein, 5mM EGTA and $100 \mu\text{M}$ verapamil (Fig. 7). In this reaction, the effect of 100nM staurosporine on the Ca^{2+} entry was not detected.

DISCUSSION

Stimulation of chemoattractant receptors causes activation of phospholipase C and A_2 , promoting

formation of inositol 1, 4, 5-trisphosphate (InsP_3), 1, 2-diacylglycerol and arachidonate (Nishizuka, 1984; Kawaguchi & Yasuda, 1986). These mediators are responsible for the release of Ca^{2+} from intracellular stores and the activation of Ca^{2+} -dependent protein kinase C. The elevation of cytosolic Ca^{2+} concentration and the activation of protein kinase C are considered to be an important factor in the stimulation of neutrophil responses (Smolen et al, 1981; Sha'afi et al, 1983). The cellular effects of elevated intracellular Ca^{2+} , including secretion and motility, are mediated by Ca^{2+} /calmodulin-dependent protein phosphorylation catalyzed by Ca^{2+} /calmodulin-dependent protein kinases (Barritt, 1992). It has been shown that calmodulin inhibitors, chlorpromazine and trifluoperazine inhibit respiratory burst and degranulation in activated phagocytic cells (Cohen et al, 1980; Lee et al, 1993). Imipramine appears to have an inhibitory action on calmodulin system. Imipramine also inhibited superoxide and H_2O_2 production in degraded IgG-activated neutrophils and depressed Ca^{2+} transport at synaptosomes (data not shown).

C5a effectively stimulated respiratory burst and lysosomal enzyme release in neutrophils. Chlorpromazine, trifluoperazine, imipramine, staurosporine and genistein inhibited superoxide production in C5a-activated neutrophils. PMA is thought to stimulate neutrophils by direct activation of protein kinase C bypass generation of inositol phosphates and resultant elevation of intracellular Ca^{2+} (Sha'afi et al, 1983; Tauber, 1987). PMA-stimulated superoxide production was inhibited by chlorpromazine, imipramine and staurosporine (Fig. 3). Thus, chlorpromazine and imipramine appear to inhibit protein kinase C. This finding supports that calmodulin inhibitors, including chlorpromazine, inhibit the activity of protein kinase C indirectly (Hidaka & Kobayashi, 1993). Chlorpromazine, trifluoperazine, imipramine, EGTA and verapamil all inhibited superoxide and H_2O_2 production in C5a-activated neutrophils. The stimulatory effect of C5a on the respiratory burst was inhibited by staurosporine and genistein. These findings indicate that receptor-linked G proteins, protein kinase C and Ca^{2+} -calmodulin system may be involved in activation of neutrophils by C5a. Inhibitory effect of genistein on the respiratory burst suggests the participation of tyrosine kinase in cellular

activation.

Effect of calmodulin inhibition on the secretion of lysosomal enzymes, such as myeloperoxidase, from activated neutrophils was investigated. C5a-induced release of lysosomal enzymes was inhibited by chlorpromazine, trifluoperazine and genistein, while imipramine and staurosporine did not show any significant effect. Thus, these results suggest that Ca^{2+} /calmodulin complex and protein tyrosine kinase is involved in activation of degranulation process. In contrast to superoxide production, degranulation is not regulated by protein kinase C. No effect of imipramine may be associated with its relatively weak inhibitory action on Ca^{2+} /calmodulin complex.

Activation of neutrophils by cell surface agonists causes Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane (Pozzan et al, 1983; Westwick & Poll, 1986). It has been demonstrated that the release of Ca^{2+} from 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 (Iino, 1991; Zhang et al, 1993). Cytosolic Ca^{2+} concentration was reached instantaneously to the maximum level after the addition of C5a. The regulatory action of calmodulin on intracellular Ca^{2+} mobilization in neutrophils were examined. C5a-elicited elevation of $[\text{Ca}^{2+}]_i$ was inhibited by chlorpromazine, imipramine, staurosporine, genistein, EGTA and verapamil but was not affected by pertussis toxin. These findings indicate that Ca^{2+} /calmodulin complex and protein kinases may be involved in the elevation of $[\text{Ca}^{2+}]_i$ in C5a-activated neutrophils. The elevation of $[\text{Ca}^{2+}]_i$ in C5a-stimulated neutrophils is probably mediated by the pertussis toxin-insensitive G proteins.

In Ca^{2+} free media, C5a induced the release of Ca^{2+} from intracellular stores. The C5a-induced Ca^{2+} release was not inhibited by chlorpromazine and imipramine. Previous report suggests that trifluoperazine potentiates Ca^{2+} -induced Ca^{2+} release in sarcoplasmic reticulum in skinned fibers of rabbit muscle (Ikemoto et al, 1996). Thus, in neutrophils intracellular Ca^{2+} release may not be regulated by Ca^{2+} /calmodulin complex. The regulatory mechanism involved in Ca^{2+} entry across the neutrophil membrane has not been clearly elucidated. It is reported that Ca^{2+} influx in gra-

nulocytes may not involve voltage-operated, receptor-operated or second messenger-operated Ca^{2+} channels (Jaconi et al, 1993). Some reports suggest the involvement of tyrosine kinases in the control of Ca^{2+} entry in platelets (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). In C5a-activated neutrophils, Mn^{2+} influx was inhibited by chlorpromazine and imipramine. The Ca^{2+} /calmodulin system appears to be involved in Ca^{2+} influx in C5a-activated neutrophils. Thapsigargin, an inhibitor of the endomembraneous Ca^{2+} ATPase, depletes intracellular Ca^{2+} stores independently of inositol phosphate formation (Jackson et al, 1988; Sargeant et al, 1993). Thapsigargin is thought to activate tyrosine kinase activity, leading to enhanced tyrosine phosphorylation and promote Ca^{2+} entry (Vostal et al, 1991; Sargeant et al, 1993). In thapsigargin-treated neutrophils, capacitance Ca^{2+} entry was initiated by adding Ca^{2+} extracellularly. The thapsigargin-evoked $[\text{Ca}^{2+}]_i$ rise was inhibited by chlorpromazine, trifluoperazine, imipramine, verapamil, and genistein but not affected by staurosporine. These results suggest that the store regulated- Ca^{2+} entry is regulated by Ca^{2+} /calmodulin system and protein tyrosine kinase. Meanwhile, it seems unlikely that capacitance Ca^{2+} entry is regulated by protein kinase C.

REFERENCES

- Barritt GJ. Calcium. In: Barritt GJ ed, *Communication within animal cells*. Oxford University Press, New York, p193–229, 1992
- Becker EL, Kermod JC, Naccache PH, Yassin R, Marsh ML, Munoz JJ, Sha'afi RI. The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. *J Cell Biol* 100: 1641–1646, 1985
- Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature* 361: 315–325, 1993
- Burt HM, Jackson JK, Salari H. Inhibition of crystal-induced neutrophil activation by a protein tyrosine kinase inhibitor. *J Leukoc Biol* 55: 112–119, 1994
- Campbell AK, Hallett MB. Measurement of intracellular calcium ions and oxygen radicals in polymorphonuclear leucocyte erythrocyte ghost hybrids. *J Physiol* 338: 537–550, 1983
- Cohen HJ, Chovaniec ME. Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for a continuous assay for monitoring superoxide production and for the study of the activation of the generating system. *J Clin Invest* 61: 1081–1087, 1978
- Cohen HJ, Chovaniec ME, Ellis SE. Chlorpromazine inhibition of granulocyte superoxide production. *Blood* 56: 23–29, 1980
- Demaurex N, Lew DP, Krause K-H. 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
- Fridovich I. Superoxide dismutases. *Annu Rev Biochem* 44: 147–159, 1975
- Goldstein IM. Complement. Biologically active products. In: Gallin JI, Goldstein IM, Synderman R ed. *Inflammation: basic principles and clinical correlates*. 2nd ed. Raven Press, Ltd., New York, p65–80, 1992
- Goldstein IM, Roos D, Weissmann G, Kaplan H. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J Clin Invest* 56: 1155–1163, 1975
- Hidaka H, Kobayashi R. Use of protein (serine/ threonine) kinase activators and inhibitors to study protein phosphorylation in intact cells. In: Hardie DG ed, *Protein phosphorylation. A practical approach*. Oxford University Press Inc., New York, p87–107, 1993
- Iannone MA, Wolberg G, Zimmerman TP. Ca^{2+} ionophore-induced cyclic adenosine-3', 5'-monophosphate elevation in human neutrophils. A calmodulin-dependent potentiation of adenylate cyclase response to endogenously produced adenosine: comparison to chemotactic agents. *Biochem Pharmacol* 42: S105–S111, 1991
- Iino M. Effects of adenine nucleotides on inositol 1,4,5-trisphosphate-induced calcium release in vascular smooth muscle cells. *J Gen Physiol* 98: 681–698, 1991
- Ikemoto T, Iino M, Endo M. Enhancing effect of calmodulin on Ca^{2+} -induced Ca^{2+} release in the sarcoplasmic reticulum of rabbit skeletal muscle fibres. *J Physiol* 487: 573–582, 1995
- Ikemoto T, Iino M, Endo M. Effect of calmodulin antagonists on calmodulin-induced biphasic modulation of Ca^{2+} -induced Ca^{2+} release. *Br J Pharmacol* 118: 690–694, 1996
- Jackson TR, Patterson SI, Thastrup O, 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
- Jaconi MEE, Lew DP, Monod A, Krause K-H. The regulation of store-dependent Ca²⁺ influx in HL-60 granulocytes involves GTP-sensitive elements. *J Biol Chem* 268: 26075–26078, 1993
- Kawaguchi H, Yasuda H. Effect of platelet-activating factor on arachidonic acid metabolism in renal epithelial cells. *Biochim Biophys Acta* 875: 525–534, 1986
- Lee CS, Kim YJ, Shin YK, Lee KS. Role of calcium and protein kinase C in platelet activating factor-induced activation of peritoneal macrophages. *Korean J Pharmacol* 29: 107–120, 1993
- Luscinskas FW, Nicolaou KC, Webber SE, Veale CA, Gimbrone MAJr, Serhan CN. Ca²⁺ mobilization with leukotriene A₄ and epoxytetraenes in human neutrophils. *Biochem Pharmacol* 39: 355–365, 1990
- Markert M, Andrews PC, Babior BM. Measurement of O₂⁻ production by human neutrophils, the preparation and assay of NADPH oxidase-containing particles from human neutrophils. In: Packer L ed, *Methods Enzymol.* 105, Academic Press Inc, p 358–365, 1984
- Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* 308: 693–698, 1984
- Painter RG, Sklar LA, Jesaitis AJ, Schmitt M, Cochrane CG. Activation of neutrophils by N-formyl chemotactic peptides. *Fed Proc* 43: 2737–2742, 1984
- Parys JB, Missiaen L, De Smedt H, Casteels R. Loading dependence of inositol 1, 4, 5-trisphosphate-induced Ca²⁺ release in the clonal cell line A7r5. Implications for the mechanism of quantal Ca²⁺ release. *J Biol Chem* 268: 25206–25212, 1993
- Pozzan T, Lew DP, Wollheim CB, Tsien RY. Is cytosolic ionised calcium regulating neutrophil activation. *Science* 221: 1413–1415, 1983
- Root RK, Metcalf J, Oshino N, Chance B. H₂O₂ release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J Clin Invest* 53: 945–955, 1975
- Sargeant P, Farndale RW, Sage SO. ADP-and thapsigargin-evoked Ca²⁺ entry and protein-tyrosin 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
- Schatzman RC, Wise CB, Kuo JF. Phospholipid-sensitive calcium-dependent protein kinase: inhibition by antipsychotic drugs. *Biochem Biophys Res Commun* 98: 669–676, 1981
- Sha'afi RI, White JR, Molski TFP, Shefeyk J, Volpi M, Naccache PH, Feinstein MB. Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. *Biochem Biophys Res Commun* 114: 638–645, 1983
- Smolen JE, Korchak HM, 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
- Spangrude GJ, Sacchi F, Hill HR, Van Epps DE, Daynes RA. Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin. *J Immunol* 135: 4135–4143, 1985
- Tauber AI. Protein kinase C and the activation of the human neutrophil NADPH-oxidase. *Blood* 69: 711–720, 1987
- Vostal JG, Jackson WL, Shulman NR. Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *J Biol Chem* 266: 16911–16916, 1991
- Westwick J, Poll C. Mechanisms of calcium homeostasis in the polymorphonuclear leukocyte. *Agents and Actions* 19: 80–86, 1986
- Wise BC, Glass DB, Jen Chou C-H, Raynor RL, Katoh N, Schatzman RC, Turner RS, Kibler RF, Kuo JF. Phospholipid-sensitive Ca²⁺-dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. *J Biol Chem* 257: 8489–8495, 1982
- Zhang B-X, Zhao H, Muallem S. Ca²⁺-dependent kinase and phosphatase control 1, 4, 5-trisphosphate-mediated Ca²⁺ release. *J Biol Chem* 268: 10997–11001, 1993
-