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# Involvement of Protein Tyrosine Kinase in Stimulated Neutrophil Responses by Sodium Fluoride

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**Abstract :** In this study, during the activation of neutrophil responses by sodium fluoride, involvement of protein tyrosine kinase was studied. Respiratory burst, lysosomal enzyme release and elevation of $[Ca^{2+}]_i$ stimulated by sodium fluoride in neutrophils were inhibited by protein kinase inhibitors, genistein and tyrphostin. The inhibitory effect of genistein and tyrphostin on superoxide and $H_2O_2$ production was less than that of protein kinase C inhibitors, staurosporine and H-7. Staurosporine and H-7 had little or no effect on the release of myeloperoxidase and acid phosphatase stimulated by sodium fluoride. EGTA and verapamil inhibited the elevation of $[Ca^{2+}]_i$ evoked by sodium fluoride. The inhibitory effect of staurosporine on the elevation of $[Ca^{2+}]_i$ was less than that of genistein. Phorbol 12-myristate 13-acetate (PMA)-stimulated superoxide production, which is sensitive to staurosporine, was further enhanced by genistein, whereas the stimulatory action of PMA on myeloperoxidase release was inhibited by genistein. A pretreatment of neutrophils with PMA signifcantly attenuated sodium fluoride-evoked elevation of $[Ca^{2+}]_i$. These results suggest that protein tyrosine kinase may be involved in the activation process of neutrophil responses due to direct stimulation of guanine nucleotide regulatory proteins. In neutrophil responses, PMA-stimulated neutrophils appear to show a different type of inhibition of protein tyrosine kinase.
**Key words :** neutrophil responses, protein tyrosine kinase, sodium fluoride.

Stimulation of neutrophils by chemoattractants results in activation of responses, including respiratory burst and degranulation (Babior, 1978; Fantone and Ward, 1982). Binding of chemoattractants to receptors on the plasma membrane causes phospholipid inositol turnover (Bareis, *et al.*, 1982), elevation of intracellular calcium (Westwick and Poll, 1986), and tyrosine phosphorylation (Berkow and Dodson, 1990), and these changes are followed by activated responses. The secretory products play an important role in host defense mechanisms and inflammatory responses (Fantone and Ward, 1982). Neutrophils are also thought to be involved in tissue destruction in inflammatory diseases, such as rheumatoid arthritis and myocardial reperfusion injury (Weiss, 1989).

Sodium fluoride induces neutrophil superoxide production after a prolonged lag period (Curnutte *et al.*, 1979; English *et al.*, 1987). Sodium fluoride has been shown to exert its action by activation of guanine nucleotide regulatory proteins (English *et al.*, 1989). Neutrophil's exposure to sodium fluoride leads to phospholipid hydrolysis and $Ca^{2+}$ influx across the plasma membrane (Blackmore *et al.*, 1985; Cockcroft and Taylor, 1987), and then cellular responses, such as superoxide production, follow.

PMA, a direct activator of protein kinase C,-induced superoxide production is inhibited by H-7 and staurosporine, inhibitors of protein kinase C, but is further stimulated by genistein (Tanimura *et al.*, 1992; Ha and Lee, 1995). Thus, superoxide production due to direct activation of protein kinase C may be affected oppositely by the change of protein tyrosine kinase activity.

In this study, to examine regulatory action of protein tyrosine kinase in neutrophil responses activated by sodium fluoride, effects of inhibitors of protein tyrosine kinase, genistein and tyrphostin on respiratory burst, lysosomal enzyme release, and intracellular $Ca^{2+}$ mobilization in sodium fluoride-stimulated neutrophils were investigated. Effects of the inhibitors were also observed in PMA-stimulated neutrophil responses.

## Materials and Methods

Sodium fluoride, phorbol 12-myristate 13-acetate (PMA), staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine dihydrochloride (H-7), genistein, tyrphostin, ethylene glycol-bis(β-aminoethyl ether)N,N,N',

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N',-tetraacetic acid (EGTA), verapamil, ferricytochrome c, scopoletin, o-dianisidine hydrochloride, a diagnostic kit for acid phosphatase, cytochalasin B, fura-2/AM and Ficoll-Hypaque solution were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents were of an 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-Hypaque density centrifugation (Markert *et al.*, 1984). The neutrophils were suspended in Dulbecco's phosphate-buffered saline at a concentration of $1\times10^7$/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 assays for the respiratory burst and degranulation were carried out.

**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 $2\times10^6$ neutrophils, 75 μM ferricytochrome c, sodium fluoride, 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 at 37°C. The reactions were initiated by the addition of sodium fluoride. Absorbance of reduced cytochrome c was continuously measured at a wavelength of 550 nm using a BECKMAN DU-70 UV-visible spectrophotometer.

**Assay of hydrogen peroxide production**

$H_2O_2$ produced from activated neutrophils was measured by the change of scopoletin fluorescence. The reaction mixtures contained $2\times10^6$ neutrophils, 2.5 μM scopoletin, 5μg/ml horse radish peroxidase, sodium fluoride, 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°C with inhibitors, the reaction was initiated by the addition of sodium fluoride. 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 $5\times10^6$/ml neutrophils in HBSS buffer with or without inhibitors were stimulated by adding sodium fluoride at 37°C. After 15 min of incubation, 250 μl of 0.2 M phosphate buffer, pH 6.2 and 250μl 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 250μl of 1% sodium azide. The absorbance was read at 450 nm (Spangrude *et al.*, 1985).

**Assay of acid phosphatase activity**

A released amount of acid phosphatase from activated neutrophils was measured using a Sigma diagnostic kit. The reaction mixtures contained $2\times10^6$ neutrophils, sodium fluoride, 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°C, the reaction mixtures were centrifuged at 3,000 rpm for 10 min, and the supernatants were taken. 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 the mU/$2\times10^6$ cells.

**Assay of cytosolic free calcium**

Fura-2 loading and fluorescence measurement were performed by the method of Luscinskas *et al.* (1990). Neutrophils (approximately $5\times10^7$ 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 was 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 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°C for 5 min with compounds, the response was initiated by the addition of sodium fluoride. 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.

**Data analysis**

The results obtained in various experiments were an-

alysed for the level of significance using the Student's *t*-test

## Results

### Effects of protein tyrosine kinase inhibitors on the stimulated respiratory burst

The respiratory burst in neutrophils was stimulated by sodium fluoride (20 mM) after approximately 5 min of lag time. The effect of protein tyrosine kinase inhibition on superoxide and $H_2O_2$ production stimulated by sodium fluoride was studied. Preincubation with either protein tyrosine kinase inhibitors, 10 μM genistein and 20 μM tyrphostin or protein kinase C inhibitors, 100 nM staurosporine and 50 μM H-7 inhibited superoxide production caused by sodium fluoride (Fig. 1). The inhibitory effects of protein tyrosine kinase inhibitors were less than those of protein kinase C inhibitors.

Hydrogen peroxide production, which is attained from the dismutation of superoxide anion ($O_2^-$) (Fridovich, 1975), was measured by the oxidation of scopoletin. Oxidation of scopoletin by neutrophils was stimulated by sodium fluoride. Change of scopoletin fluorescence occured approximately 8 min post-addition of sodium fluoride (20 mM). Involvement of protein tyrosine kinase in $H_2O_2$ production stimulated by sodium fluoride was examined. Fig. 2 shows that 10 μM genistein, 20 μM tyrphostin and 100 nM staurosporine inhibited $H_2O_2$ production caused by sodium fluoride. The inhibitory effects of genistein and tyrpostin were less than that of staurosporine, and they showed a little inhibitory effect. The degree of inhibitory effects of protein kinase inhibitors on $H_2O_2$ production was similar to that on superoxide production.

Effects of protein kinase inhibitors on sodium fluoride-induced superoxide production were compared with the stimulatory effect of PMA. As shown in Fig. 3, in contrast to sodium fluoride the stimulated superoxide production by 0.1 μg/ml PMA, a direct activator of protein kinase C, was further enhanced by the addition of 10 μM genistein, while the stimulatory effect of PMA was markedly inhibited by 100 nM staurosporine.

### Inhibition of sodium fluoride-stimulated lysosomal enzyme release by protein tyrosine kinase inhibitors

The secretion of lysosomal enzymes from activated neutrophils was assayed by measuring the release of

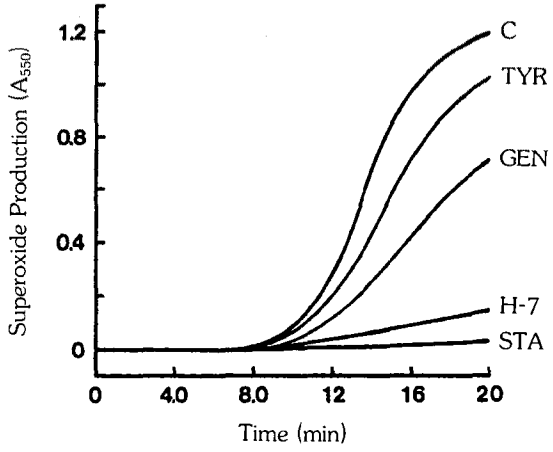


**Fig. 1.** Inhibitory effects of protein kinase inhibitors on superoxide production in neutrophils activated by sodium fluoride. Neutrophils ($2\times10^6$ cells/ml) were stimulated with 20 mM sodium fluoride in the presence of inhibitors. C, no addition; GEN, 10 μM genistein; TYR, 20 μM tyrphostin; STA, 100 nM staurosporine; H-7, 50 μM H-7. The traces are representative of three experiments.

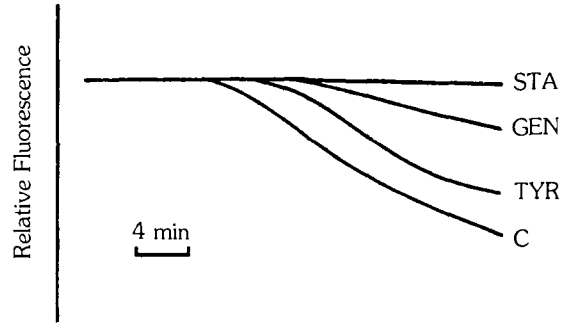


**Fig. 2.** Inhibition of sodium fluoride-induced hydrogen peroxide production by the kinase inhibitors. Neutrophils ($2\times10^6$ cells/ml) were stimulated with 20 mM sodium fluoride in the presence of 10 μM genistein (GEN), 20 μM tyrphostin (TYR) and 100 nM staurosporine (STA) and or not (C). The traces are representative of three experiments.

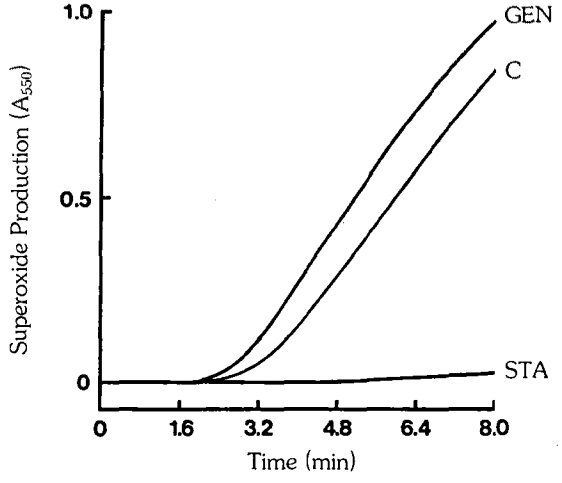


**Fig. 3.** Effects of protein kinase inhibitors on superoxide production stimulated by PMA. Neutrophils ($2\times10^6$ cells/ml) were stimulated with 0.1 μg/ml PMA (C) in the presence of 10 μM genistein (GEN) or 100 nM staurosporine (STA). The traces are representative of three experiments.

myeloperoxidase and acid phosphatase. The effects of protein kinase inhibitors on sodium fluoride-induced release of lysosomal enzymes were investigated. Table 1 shows that 10 μM genistein and 20 μM tyrphostin significantly inhibited the release of myeloperoxidase and acid phosphatase stimulated by 20 mM sodium fluoride, whereas 100 nM staurosporine had no effect on lysosomal enzyme release. In this reaction, sodium fluoride-induced myeloperoxidase release was slightly inhibited by 50 μM H-7. The inhibitory pattern of protein kinase inhibitors on lysosomal enzyme release did not coincide with that on the respiratory burst.

The stimulatory effect of 0.1 μg/ml PMA on myeloperoxidase release was inhibited by both 10 μM genistein and 100 nM staurosporine (Fig. 4). In contrast to superoxide production, the myeloperoxidase release induced by PMA was not stimulated by genistein.

**Table 1.** Effects of protein kinase inhibitors on lysosomal enzyme release

| Compounds | Myeloperoxidase (nmol/5×10⁶cells) | Acid phosphatase (mU/4×10⁶ cells) |
|---|---|---|
| no addition | 10.38±2.35 | 255.52±37.87 |
| +10 μM Genistein | 1.48±0.58[a] | 147.59±11.79[a] |
| +20 μM Tyrphostin | 3.44±0.83[a] | |
| +100 nM Staurosporine | 9.78±0.73 | 223.64±16.57 |
| +50 μM H-7 | 7.98±1.23 | - |

After 5 min of preincubation with inhibitors, the release of myeloperoxidase and acid phosphatase from neutrophils were initiated by the addition of 20 mM sodium fluoride. Values are means ± S.D., n=5. [a]$p < 0.01$ by Student's *t*-test.

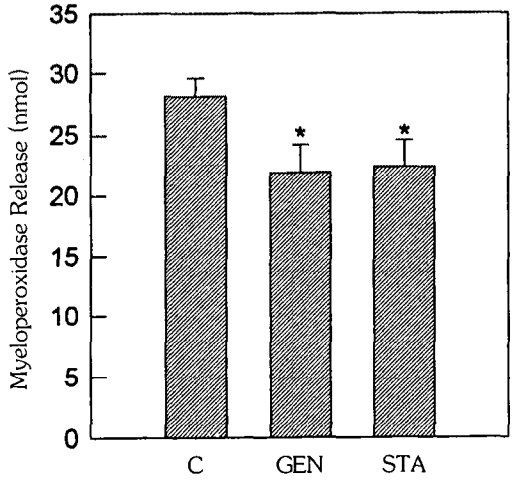


**Fig. 4.** Effects of protein kinase inhibitors on PMA-induced myeloperoxidase release. Neutrophils ($5\times10^6$ cells/ml) were stimulated with 0.1 μg/ml PMA in the presence of inhibitors or not (C). Values are means ± S.D., n=4. GEN, 10 μM genistein; STA, 100 nM staurosporine. *$p<0.05$ by Student's *t*-test.

**Effects of protein tyrosine kinase inhibitors on intracellular calcium mobilization**

The elevation of intracellular calcium ($[Ca^{2+}]_i$) is attained by both release of $Ca^{2+}$ from the intracellular stores and $Ca^{2+}$ influx across the plasma membrane (Westwick and Poll, 1986). The elevation of $[Ca^{2+}]_i$ in neutrophils occured at approximately 1 min post-addition of 20 mM sodium fluoride. The $[Ca^{2+}]_i$ was gradually increased with time and was reached at a maximum level after 5 min of incubation. After the addition of sodium fluoride, the lag time in the elevation of $[Ca^{2+}]_i$ was significantly shorter than that in the respiratory burst. The effect of protein kinase inhibition on sodium fluoride-induced elevation of $[Ca^{2+}]_i$ was studied. A preincubation of fura-2-loaded neutrophils with either 10

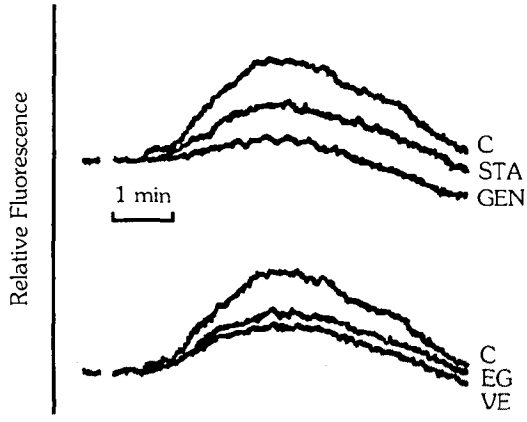


**Fig. 5.** Effects of the kinase inhibitors on the elevation of $[Ca^{2+}]_i$ evoked by sodium fluoride. Fura-2-loaded neutrophils ($4\times10^6$ cells/ml) were preincubated with inhibitors for 5 min, and then the response was initiated by the addition of 20 mM sodium fluoride. C, no addition; GEN, 10 μM genistein; STA, 100 nM staurosporine; EG, 5 mM EGTA; VE, 100 μM verapamil. The traces are representative of three experiments.

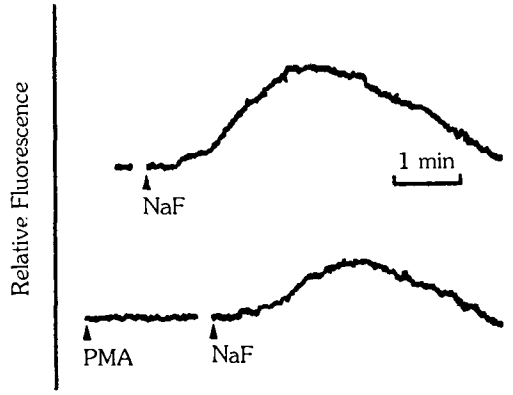


**Fig. 6.** Inhibition of sodium fluoride-induced elevation of $[Ca^{2+}]_i$ by pretreatment of PMA. Fura-2-loaded neutrophils ($4\times10^6$ cells/ml) was treated with 0.1 μg/ml PMA, and then 20 mM sodium fluoride was added to induce elevation of $[Ca^{2+}]_i$ sequentially.

μM genistein or 100 nM staurosporine inhibited the elevation of $[Ca^{2+}]_i$ evoked by 20 mM sodium fluoride (Fig. 5). The sodium fluoride-induced elevation of $[Ca^{2+}]_i$ was inhibited by 5 mM EGTA, a $Ca^{2+}$ chelator or 0.1 mM verapamil, a $Ca^{2+}$ channel blocker.

The activation of protein kinase C is thought to attenuate chemoattractant-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils (McCarthy *et al.*, 1989). As shown in Fig. 6, after the pretreatment with 0.1 μg/ml PMA, the stimulatory effect of sodium fluoride on the elevation of $[Ca^{2+}]_i$ in neutrophils was significantly decreased.

## Discussion

Fluorides have been shown to affect the activity of adenylate cyclase and activate cyclic GMP phosphodiesterase (Kanaho *et al.*, 1985). They release histamine from mast cells (Kuza and Kazimierczak, 1982), stimulate respiratory burst in neutrophils (Curnutte *et al.*, 1979) and activate phosphorylase in hepatocytes (Corson and Fein, 1983). Sodium fluoride (NaF) has been shown to activate phospholipase-associated guanine nucleotide binding protein directly (English *et al.*, 1989) and then stimulate neutrophil responses, respiratory burst and lysosomal enzyme release. In contrast to chemoattractants, it shows a long time of activation phase (Snyderman and Uhing, 1992). Sodium fluoride, like GTPγS, is reported to stimulate generation of $InsP_3$ in a dose-dependent fashion (Cockcroft and Taylor, 1987). However, the role of protein tyrosine kinase in activation processes in sodium fluoride-stimulated neutrophils has not been clearly elucidated.

In this study, during the activation of neutrophil responses by direct stimulation of G proteins, the regulatory roles of protein tyrosine kinase were examined. Sodium fluoride effectively stimulates respiratory burst in neutrophils after a long lag time compared with chemoattractants. Superoxide and $H_2O_2$ production stimulated by sodium fluoride was inhibited by both protein kinase C inhibitors and protein tyrosine kinase inhibitors. The inhibitory effect of protein tyrosine inhibitors was less than that of protein kinase C inhibitors. The findings indicate that sodium fluoride appears to exert chiefly its stimulatory action on neutrophils through activation of protein kinase C after G protein stimulation. In addition, the stimulatory action of sodium fluoride may also be partly mediated by activation of protein tyrosine kinase. In contrast, the PMA-stimulated superoxide production, which is sensitive to staurosporine, was enhanced by genistein. The result coincides with previous reports (Tannimura *et al.*, 1992; Ha and Lee, 1995). The stimulated respiratory burst due to direct activation of protein kinase C may be reversibly regulated by the change of protein tyrosine kinase activity.

Protein tyrosine kinase inhibitors significantly inhibited the release of myeloperoxidase and acid phosphatase from neutrophils stimulated by sodium fluoride, whereas protein kinase C inhibitors had little or no effect on it. In contrast to the respiratory burst, the release of lysosomal enzymes appears to be mainly mediated by protein tyrosine kinase rather than protein kinase C. Myeloperoxidase release caused by direct activation of protein kinase C was inhibited by genistein. Thus, the present data support that the activation mechanism of lysosomal enzyme release may be different from that of the respiratory burst (Shin *et al.*, 1989). From the results, the regulatory roles of protein kinase C in the activation process of lysosomal enzyme release in neutrophils are not suggested.

Changes in the cytosolic free calcium level are thought to play an important role in the activation process of neutrophil responses. Elevation of $[Ca^{2+}]_i$ is an early event in the response of neutrophils to agonists, including fMLP, complement C5a and platelet-activating factor (Westwick and Poll, 1986). The receptor binding of chemoattractants 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 an influx across the plasma membrane, follows (Cobbold and Rink, 1987). Sodium fluoride evoked elevation of $[Ca^{2+}]_i$ in neutrophils after a relative long activation phase, as compared with chemoattractants which caused an immediate elevation of $[Ca^{2+}]_i$ post-addition. The lag time in the elevation of $[Ca^{2+}]_i$ was much shorter than that in the respiratory burst. The finding indicates that, after addition of sodium fluoride, the elevated cytosolic $Ca^{2+}$ may be involved in the initiation of the stimulation of neutrophil responses. Inhibition of sodium fluoride-evoked elevation of $[Ca^{2+}]_i$ by EGTA and verapamil supports the above views on intracellular $Ca^{2+}$ mobilization.

Receptor-mediated intracellular calcium mobilization is thought to be coupled to phospholipase C activation which promotes phosphoinositide hydrolysis (Berridge, 1987). The initial intracellular $Ca^{2+}$ release is mediated by InsP3. However, the mechanism underlying receptor-mediated $Ca^{2+}$ influx is uncertain. Inhibition of the elevation of $[Ca^{2+}]_i$ in sodium fluoride-stimulated neutrophils by the kinase inhibitors indicates that the sodium fluoride-evoked elevation of $[Ca^{2+}]_i$ appears to be regulated by protein tyrosine kinase and protein kinase C. In this response, the inhibitory effect of genistein was greater than that of staurosporine. It has been shown that the activation of protein kinase C with PMA attenuates agonist-evoked elevation of $[Ca^{2+}]_i$ by in-

hibition of intracellular $Ca^{2+}$ mobilization (Della Bianca *et al.*, 1986; McCarthy *et al.*, 1989). A pretreatment of fura-2-loaded neutrophils with PMA significantly inhibited the elevation of $[Ca^{2+}]_i$ evoked by sodium fluoride. The result suggests that sodium fluoride-induced stimulation of the responses could be attenuated by direct activation of protein kinase C.

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