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Comparision of Regulatory Action of cAMP and cGMP on the Activation of Neutrophil Responses

Chang-Hwang Han, Young-Chul Yoon, Yong-Kyoo Shin, Eun-Sook Han, and Chung-Soo Lee*

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

The regulatory role of cyclic nucleotides in the expression of neutrophil responses has been examined. fMLP-stimulated superoxide production in neutrophils was inhibited by dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP), histamine, adenosine+theophylline, cAMP elevating agents, and 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP) and sodium nitroprusside, cGMP elevating agents. Staurosporine, a protein kinase C inhibitor, genistein, a protein tyrosine kinase inhibitor and chlorpromazine, a calmodulin inhibitor, inhibited superoxide production by fMLP, but they did not further affect the action of DBcAMP on the stimulatory action of fMLP. DBcAMP, histamine, adenosine+theophylline and genistein inhibited myeloperoxidease release evoked by fMLP, whereas BrcGMP, sodium nitroprusside and staurosporine did not affect it. The elevation of $[Ca^{2+}]_i$ evoked by fMLP was inhibited by genistein and chlorpromazine but was not affected by staurosporine. DBcAMP exerted little effect on the initial peak in $[Ca^{2+}]_i$ response to fMLP but effectively inhibited the sustained rise. On the other hand, BrcGMP significantly inhibited both phases. fMLP-induced Mn^{2+} influx was inhibited by either DBcAMP or BrcGMP. These results suggest that fMLP-stimulated neutrophil responses may be regulated by cAMP more than cGMP. cAMP and cGMP appear not affect stimulated responses by direct protein kinase C activation. Their regulatory action on the stimulated neutrophil responses may be not influenced by other activation processes.

Key Words: Cyclic nucleotides, Protein kinase and calmodulin inhibitors, Neutrophils

INTRODUCTION

The lysosomal enzyme release from neutrophils are reported to be regulated by adrenergic and cholinergic agents. It has been suggested that agents which elevate the levels of cAMP within neutrophils inhibit the release of enzymes during feeding of zymosan particles coated with immune complexes, whereas agents which elevate the levels of cGMP within neutrophils enhance the release of enzyme (Zurier et al., 1974). Neutrophil response to external stimuli can be altered by the change of cytosolic nucleotide level. Increase of intracellular cAMP levels is associated with a decrease of neutrophil responses, chemotaxis, respiratory burst and lysosomal enzyme release (Stephens and Snyderman, 1982; Lad et al., 1985). β-Adrenergic agonists (Nielson, 1987) and histamine (Seligman et al., 1983) have been shown to inhibit neutrophil enzyme release and superoxide anion production through accumulation of cAMP by receptor-mediated stimulation of adenylyl cyclase. β-Adrenergic agonists enhance accumulation of cAMP in response to chemoattractants, such as N-formyl-methionylleucyl-phenylalanine (fMLP) (Reibman et al., 1990). cAMP level can be elevated by inhibition of a cyclic nucleotide phosphodiesterase which mediates cAMP degradation (Grady and Thomas, 1986). However, influence of intracellular elevated cGMP on chemoattractants--activated neutrophil responses are uncertain.

cAMP and cGMP are considered to inhibit activation of phospholipase C, elevation of $[Ca^{2+}]_i$, activation of protein kinase C, aggregation and secretion in platelets (Takai et al., 1982; Lazarowski and Lapetina, 1989; Pusqui et al., 1990). However, in activated neutrophils regulatory role of cAMP and cGMP in activity of phospholipase C and intracellular Ca^{2+} mobilization has not been elucidiated. The present

*To whom all correspondences should be addressed.

study was done to investigate and compare the regulatory action of elevated intracellular cAMP and cGMP in fMLP-stimulated neutrophil responses, such as superoxide production, myeloperoxidase release and intracellular Ca^{2+} mobilization. Their effects were also observed on the stimulatory actions of phorbol 12-myristate 13-acetate, a direct activator of protein kinase C. Influence of the protein kinase C and protein tyrosine kinase inhibition on the action of cyclic nucleotides in neutrophil responses was examined.

MATERIALS AND METHODS

N-Formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP), 8-bromoguanosine 3',5'-cyclic monophosphate (BrcGMP), histamine, adenosine, theophylline, sodium nitroprusside, staurosporine, genistein, chlorpromazine, cytochalasin B, ferricytochrome c, o-dianisidine and fura-2/AM 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 1×10^7 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 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, stimulating agent, 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 10 min. The reaction was then stopped by placing the tubes in melting ice, and the cells were rapidly pelleted by centrifuging at 1,500 g for 5 min at 4°C. The supernatants were taken, and 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). The absorbance changes were also traced with printer.

Assay of myeloperoxidase release

Measurement of myeloperoxidase release was done by the method of Spangrude et al. (1985). A 5×10^6/ml neutrophils in HBSS buffer with or without inhibitors were stimulated by adding fMLP (or PMA). 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 and fluorescence measurement were performed by the method of Luscinskas et al. (1990). Neutrophils (approximately 5×10^7 cells/ml) were loaded with 2 mM fura-2/AM to 1 μM/10^7 cells at 37°C for 10 min in the reaction mixtures contained HBSS buffer without calcium and magnesium (HBSS-CMF) and 20 mM HEPES-tris, pH 7.4. The suspension was then diluted 5 fold with 0.5% bovine serum albumin containing HBSS-CMF and 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 5×10^7 cells/ml. Fluorescence measurement was done with 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 1 μM fMLP. The fluorescence change was read at an excitation wavelength of 340 nm and emission wavelength of 505 nm.

Assay of Mn^{2+} influx

Influx of Mn^{2+} into cells was measured using the fura-2 fluorescence quenching technique (Demaurex

et al., 1992). Fura-2 loaded neutrophils (4×10^6/ml) were suspended in Ca^{2+}-and Mg^{2+}-containing HBSS media. After 90 sec of stimulation with fMLP, Mn^{2+} (0.5 mM) was added, and quenching of fura-2 fluorescence by Mn^{2+} influx was measured at an excitation wavelength of 360 nm and emission wavelength of 505 nm.

RESULTS

Effects of DBcAMP and 8-BrcGMP on superoxide production stimulated by fMLP

fMLP has been shown to stimulate superoxide production and lysosomal enzyme release significantly (Showell et al., 1976; Lackie, 1982). The effects of dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP) and 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP) on superoxide production in fMLP-stimulated neutrophils were examined. One μM fMLP-induced superoxide production was inhibited by 1 mM of DBcAMP and 8-BrcGMP (Fig. 1). The inhibitory effect of DBcAMP was greater than that of 8-BrcGMP.

Effect of DBcAMP on superoxide production was compared with other cyclic nucleotide elevating agents. A 100 μM histamine, 10 μM adenosine and 50 μM sodium nitroprusside inhibited superoxide production stimulated by fMLP (Fig. 2). In the presence of 100 μM theophylline, this elevated superoxide production was further inhibited by adenosine, while theophylline alone did not cause any significant effect.

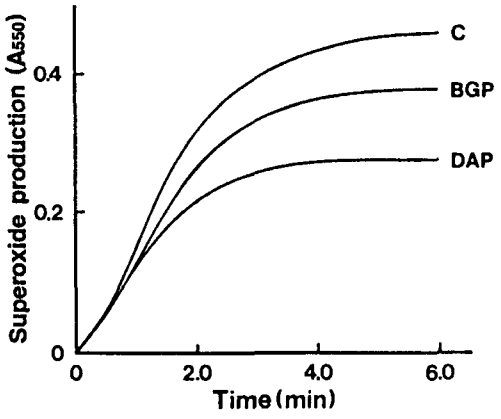

Fig. 1. Effects of cyclic nucleotide elevating agents on superoxide production in neutrophils activated by fMLP. Neutrophils (2×10^6 cells/ml) were stimulated with 1 μM fMLP in the presence of 1 mM DBcAMP (DAP) or 1 mM BrcGMP (BGP). The traces are representative of three experiments.

Effects of protein kinase inhibitors and calmodulin inhibitor on the inhibitory effect of DBcAMP

In the state of protein kinase inhibition, effects of cyclic nucleotide elevating agents on superoxide production were observed. A 100 μM staurosporine, a protein kinase C inhibitor, and 10 μM genistein, a protein tyrosine kinase inhibitor, inhibited superoxide production stimulated by fMLP. Fig. 3 shows that inhibition of superoxide production by protein kinase inhibitor was not further enhanced by the addition of DBcAMP.

Influence of calmodulin inhibition on the inhibitory action of DBcAMP was investigated. fMLP-stimulated superoxide production was markedly inhibited by 50 μM chlorpromazine. As shown in Fig. 4, the decrease of superoxide production by chlorpromazine was not further affected by the addition of DBcAMP.

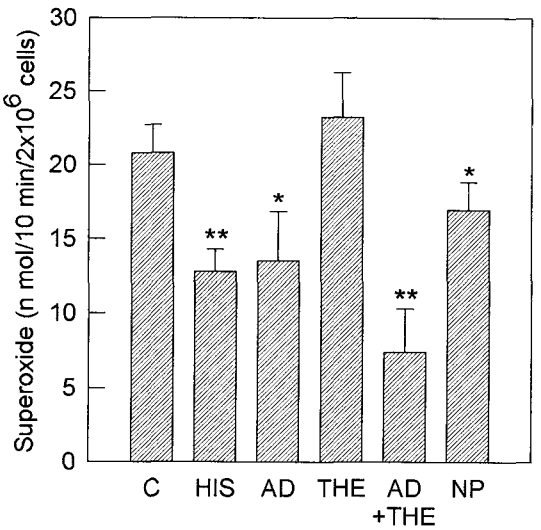

Fig. 2. Effects of cAMP and cGMP elevating agents on superoxide production in neutrophils activated by fMLP. Neutrophils (2×10^6 cells/ml) were stimulated with 1 μM fMLP in the presence of agents. Values are means $\pm$ SD, n=5. C, no addition; HIS, 100 μM histamine; THE, 100 μM theophylline; AD, 10 μM adenosine; NP, 50 μM sodium nitroprusside. $^{**}p<0.01$, $^{*}p<0.05$ by Student's *t*-test.

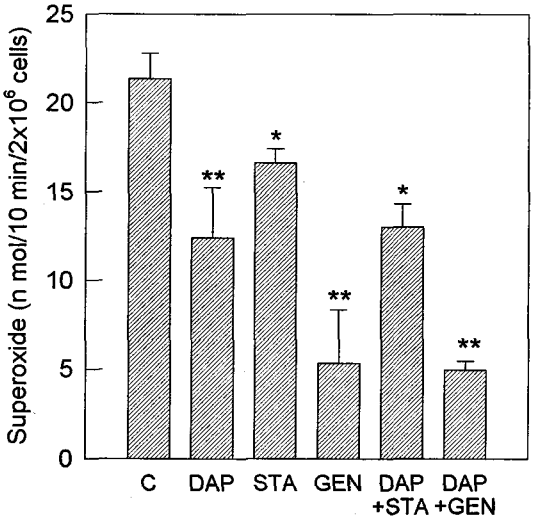

Fig 3. Effects of protein kinase inhibitors in the presence of DBcAMP on fMLP-induced superoxide production. Neutrophils were stimulated with 1 μM fMLP in the presence of 1 mM DBcAMP (DAP), 100 nM staurosporine (STA) and 10 μM genistein (GEN) or not (C). Values are means $\pm$ SD, n=5. $^{**}p<0.01$, $^{*}p<0.05$ by Student's t-test.

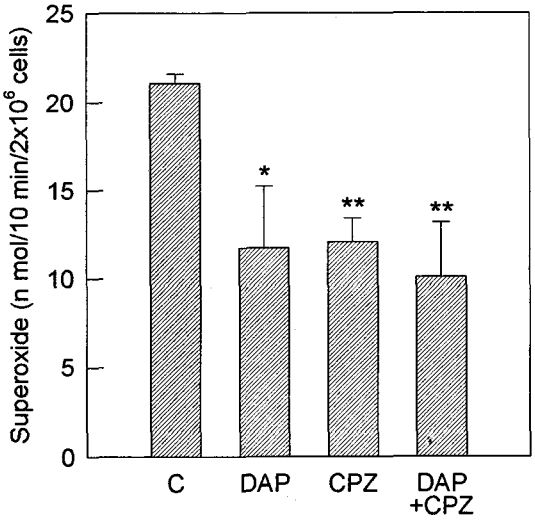

Fig. 4. Effect of calmodulin inhibitor in the presence of DBcAMP on fMLP-induced superoxide production. Neutrophils were stimulated with 1 μM fMLP in the presence of 1 mM DBcAMP (DAP) and 50 μM chlorpromazine (CPZ) or not (C). Values are means $\pm$ SD, n=5. $^{**}p<0.01$, $^{*}p<0.05$ by Student's t-test.

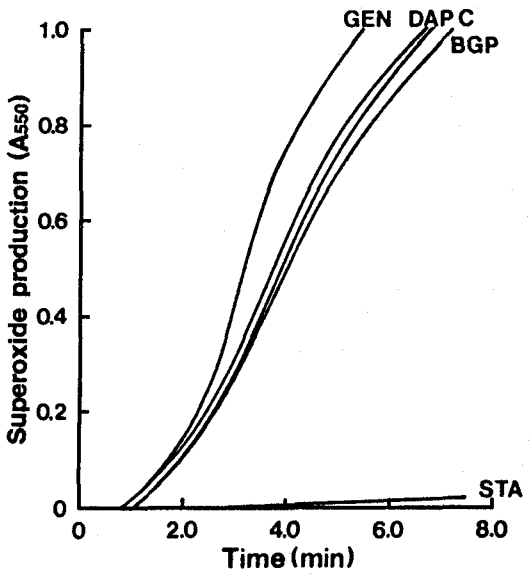

Fig. 5. Effects of cyclic nucleotide elevating agents on superoxide production in neutrophils activated by PMA. Neutrophils were stimulated with 0.1 μg/ml PMA in the presence of agents. C, no addition; DAP, 1 mM DBcAMP; BGP, 1 mM BrcGMP; STA, 100 nM staurosporine; GEN, 10 μM genistein. The traces are representative of three experiments.

Table 1. Effects of cyclic nucleotide elevating agents on myeloperoxidase release from activated neutrophils

Compounds	Myeloperoxidase release (nmol/5 $\times$ 10^6 cells)
No addition	28.69 $\pm$ 1.28
1 mM DBcAMP	15.73 $\pm$ 2.63**
100 μM Histamine	21.63 $\pm$ 1.75**
10 μM Adenosine	26.91 $\pm$ 4.22
100 μM Theophylline	27.50 $\pm$ 3.30
Adenosine + Theophylline	11.21 $\pm$ 2.87**
1 mM BrcGMP	29.19 $\pm$ 0.71
100 μM Sodium nitroprusside	28.42 $\pm$ 0.91

After 5 min of preincubation with inhibitors, neutrophils (5 $\times$ 10^6 cells/ml) were stimulated with 1 μM fMLP. Values are means $\pm$ SD, n=4-5. $^{**}p<0.01$ by Student's t-test.

Effects of DBcAMP and 8-BrcGMP on superoxide production stimulated by PMA

PMA stimulates neutrophil responses by activating protein kinase C directly (Tauber, 1987). Effects of DBcAMP and BrcGMP on PMA-stimulated superoxide production were studied. Fig. 5 shows that 0.1 μg /ml PMA-induced superoxide production was not

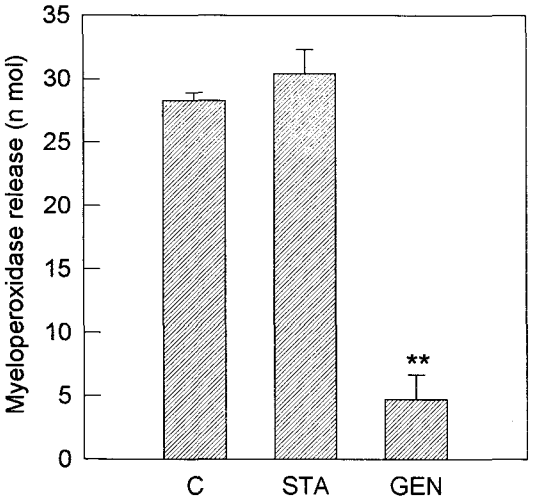

Fig. 6. Effects of protein kinase inhibitors on fMLP-induced myeloperoxidase release. Neutrophils were stimulated with 1 μM fMLP in the presence of 100 nM staurosporine (STA) and 10 μM genistein (GEN) or not (C). Values are means$\pm$SD, n=4. $^{**}p<0.01$ by Student's *t*-test.

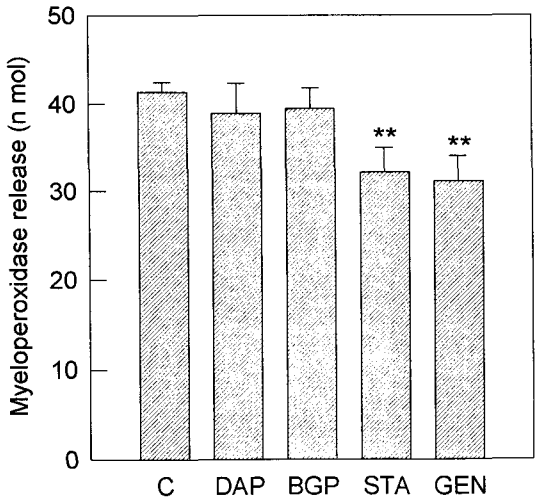

Fig. 7. Effects of DBcAMP and BrcGMP on PMA-induced myeloperoxidase release Neutrophils were stimulated with 0.1 μg/ml PMA in the presence of 1 mM DBcAMP (DAP), 1 mM BrcGMP (BGP), 100 nM staurosporine (STA) and 10 μM genistein (GEN) or not (C). Values are means$\pm$SD, n=4. $^{**}p<0.01$ by Student's *t*-test.

affectd by 1 mM DBcAMP, 1 mM BrcGMP and 100 μM histamine. Staurosporine (100 nM) inhibited superoxide production by PMA, whereas 10 μM genistein stimulated it.

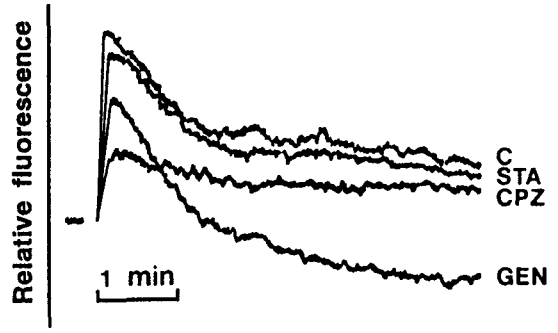

Fig. 8. Effects of protein kinase and calmodulin inhibitors on fMLP-evoked elevation of $[Ca^{2+}]_i$. Fura-2-loaded neutrophils (4×10^6 cells/ml) were preincubated with 100 nM staurosporine (STA), 10 μM genistein (GEN) and 50 μM chlorpromazine (CPZ) or not (C) for 5 min, and then the response was initiated by the addition of 1 μM fMLP. The traces are representative of three experiments.

Effects of cyclic nucleotide elevating agents on myeloperoxidase release

The secretion of lysosomal enzymes from activated neutrophils was assayed by measuring the release of myeloperoxidase. Table 1 shows that 1 mM DBcAMP and 100 μM histamine inhibited myeloperoxidase release induced by 1 μM fMLP, while 1 mM BrcGMP and 50 μM sodium nitroprusside did not affect it. Either 10 μM adenosine alone or 100 μM theophylline alone did not have any significant effect on myeloperoxidase release. However, in the simultaneous presence of adenosine and theophylline, fMLP-induced myeloperoxidase release was significantly inhibited.

Effect of protein kinase inhibition on stimulated myeloperoxidase was examined. fMLP-stimulated myeloperoxidase release was not affected by 100 nM staurosporine but was inhibited by 10 μM genistein (Fig. 6).

Influence of elevated intracellular cyclic nucleotide levels on myeloperoxidase release evoked by activation of protein kinase C. As shown in Fig. 7, 0.1 μg/ml PMA-stimulated myeloperoxidase release was not affected by 1 mM DBcAMP and 1 mM BrcGMP. Meanwhile, 100 nM staurosporine and 10 μM genistein inhibited the release induced by PMA.

Effects of protein kinase inhibitors and calmodulin inhibitors on intracellular Ca^{2+} mobilization

One μM fMLP caused an immediate increase of intracellular Ca^{2+} level ($[Ca^{2+}]_i$) in fura-2-loaded

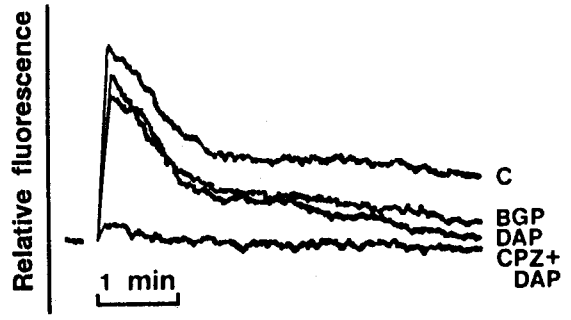

Fig. 9. Effects of DBcAMP and BrcGMP on fMLP-evoked elevation of $[Ca^{2+}]_i$. Fura-2-loaded neutrophils were preincubated with 1 mM DBcAMP (DAP), 1 mM BrcGMP (BGP) and 50 μM chlorpromazine + 1 mM DBcAMP (CPZ + DAP) or not (C). The traces are representative of three experiments.

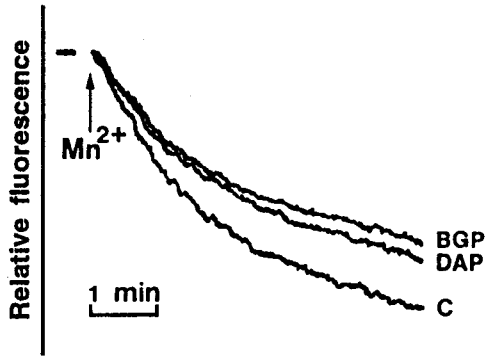

Fig. 10. Effects of DBcAMP and BrcGMP on Mn^{2+} influx induced by fMLP. Mn^{2+} influx into the cytoplasm of neutrophils were initiated by adding 0.5 mM Mn^{2+} after 90 sec of stimulation with 1 μM fMLP. Fura-2-loaded neutrophils were stimulated with 1 μM fMLP in the presence of 1 mM DBcAMP (DAP) and 1 mM BrcGMP (BGP) or not (C). The traces are representative of three experiments.

neutrophils in 1.23 mM Ca^{2+}-containing medium, and the elevated $[Ca^{2+}]_i$ was gradually decreased over the subsequent several minutes. The effect of protein kinase inhibition on fMLP-induced elevation of $[Ca^{2+}]_i$ was investigated. A preincubation of fura-2-loaded neutrophils with 100 nM staurosporine did not affect the elevation of $[Ca^{2+}]_i$ evoked by 1 μM fMLP (Fig. 8). However, 10 μM genistein had an inhibitory effect on the stimulatory action of fMLP.

Addition of 50 μM chlorpromazine markedly inhibited the elevation of $[Ca^{2+}]_i$ evoked by fMLP.

Inhibition of fMLP-induced elevation of $[Ca^{2+}]_i$ by DBcAMP and BrcGMP

Effect of elevated cyclic nucleotide levels on fMLP-induced intracellular Ca^{2+} mobilization was studied. Fig. 9 shows that the initial peak of $[Ca^{2+}]_i$ in responses to fMLP was slightly decreased by 1 mM DBcAMP. And DBcAMP inhibited the sustained elevation of $[Ca^{2+}]_i$ after the stimulation. Meanwhile, a preincubation with 1 mM BrcGMP inhibited both initial peak and sustained elevation of $[Ca^{2+}]_i$ in response to fMLP.

The stimulatory action of fMLP on $[Ca^{2+}]_i$ was almost completely inhibited by 1 mM DBcAMP plus 50 μM chlorpromazine.

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. 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, fMLP did not cause fluorescence change in fura-2-loaded neutrophils. When added to 1 μM fMLP-stimulated neutrophils, 0.5 mM Mn^{2+} caused an immediate and continuous decrease in fluorescence. Influence of DBcAMP and BrcGMP on Ca^{2+} influx across the plasma membrane was examined. Neutrophils were preincubated with either DBcGMP or BrcGMP for 5 min and then were exposed to 1 μM fMLP for 90 sec prior to Mn^{2+} addition. fMLP-induced Mn^{2+} influx was inhibited by 1 mM of DBcAMP and BrcGMP (Fig. 10).

DISCUSSION

Elevation of intracellular cAMP level causes inhibition of the respiratory burst, degranulation and phospholipid inositol turnover cytosolic Ca^{2+} level in neutrophils (Lad et al., 1985; Takenawa et al., 1986). Human neutrophils posses $G_{\alpha s}$-linked receptors, and activation of the receptors stimulates cAMP production (Nielson and Vestal, 1989). fMLP has been shown to increase intracellular cAMP level indirectly by a mechanism requiring Ca^{2+} mobilization without direct stimulation of adenylyl cyclase (Verghese et al., 1985). In contrast, β-adrenergic agonists and histamine cause elevation of intracellular cAMP level by stimulation of adenylyl cyclase (Nielson, 1987) and promote the

accumulation of cAMP in response to fMLP (Reibman et al., 1990). They inhibit neutrophil respiratory burst and lysosomal enzyme release stimulated by chemoattractants. On the other hand, cGMP has been reported to inhibit aggregation and tyrosine phosphorylation in platelets (Oda et al., 1992). However, the regulatory role of cGMP in activated neutrophil responses has not been elucidated.

The neutrophil superoxide production in response to fMLP was suppressed by DBcAMP and 8-BrcGMP. fMLP-induced superoxide production was also inhibited by histamine, adenosine plus theophylline and sodium nitroprusside. In the presence of cyclic nucleotide phosphodiesterase inhibitor, adenosine elicits an increase of intracellular cAMP level (Cronstein et al., 1988). Thus, elevated intracellular cAMP and cGMP may exhibit an inhibitory effect on the respiratory burst stimulated by fMLP. And superoxide production stimulated by fMLP may be less sensitive to cGMP. Protein kinase C, protein tyrosine kinase and Ca^{2+} /calmodulin complex appear to be involved in stimulation of the respiratory burst in neutrophils activated by fMLP. cGMP has been reported to affect protein kinase C activity and inhibit tyrosine phosphorylation. Thus, it is possible that cAMP may affect the other activation processes. The inhibitory effect of cAMP on superoxide production was not affected by inhibition of protein kinase C, protein tyrosine kinase and Ca^{2+}/calmodulin complex. Regulatory actions of cAMP and cGMP on stimulated respiratory burst appear to be not affected by other activation processes. The stimulatory action of genistein on the PMA effect suggests that the protein kinase C-mediated neutrophil responses could be regulated by protein tyrosine kinase system oppositely.

The stimulated neutrophil myeloperoxidase release by fMLP was inhibited by DBcAMP, histamine, adenosine plus theophylline and genistein but was not affectd by 8-BrcGMP, sodium nitroprusside and staurosporine. Agonist-induced lysosomal enzyme release may be affected by elevated cAMP and activated tyrosine kinase. In contrast superoxide production, in the process of degranulation the regulatory roles of cGMP and protein kinase C are not suggested. This finding also partly supports that the activation process of degranulation appears to be different from the respiratory burst (Sim et al., 1992). As in superoxide production, PMA-stimulated myelopeloxidase release, which is responsible for the protein kinase inhibitors, was not affected by DBcAMP and 8-BrcGMP. Influences of cyclic nucleotide elevating agents on neutrophil responses stimulated by PMA were similar to those of adenosine and adenosine analogues (data not shown).

A changes in cytosolic free calcium level are thought to play an important role in the activation process of neutrophil responses. 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).

fMLP caused an immediate increase of $[Ca^{2+}]_i$ in fura-2-loaded neutrophils. The elevated $[Ca^{2+}]_i$ was gradually decreased. Receptor-mediated intracellular Ca^{2+} mobilization is though to be coupled to phospholipase C activation which promotes phosphoinositide hydrolysis with the formation of Inositol 1,4,5--trisphosphate ($InsP_3$) and 1,2-diacylglycerol (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. Inositol 1,3,4,5,-tetrakisphosphate ($InsP_4$) may be responsible for the activation of Ca^{2+} influx (Irvine and Moor, 1986). Activation of fMLP receptors evoked both intracellular Ca^{2+} release and Ca^{2+} entry across the plasma mambrane. Thus, this finding supports the above view on the intracellular Ca^{2+} mobilization.

Influences of elevated intracellular cyclic nucleotides on intracellular Ca^{2+} mobilization, which may be regulated by protein tyrosine kinase and Ca^{2+}/calmodulin system, were investigated. DBcAMP exerted little effect on the initial peak in $[Ca^{2+}]_i$ in response to fMLP but effectively inhibit the sustained rise. On the other hand, BrcGMP significantly inhibited both phases. The divalent cation Mn^{2+} has been shown to permeate through Ca^{2+} influx pathway in neutrophils (Demaurex et al., 1992; Jaconi et al., 1993). The inhibition of fMLP-induced Mn^{2+} influx by DBcAMP or BrcGMP indicates that the sustained rise appears to be affected by changes of intracellular cAMP or cGMP. Cyclic nucleotides may regulate intracellular Ca^{2+} mobilization in fMLP-activated neutrophils by their effects on intracellular Ca^{2+} release and Ca^{2+} influx across the plasma membrane.

Compared with the significant effect of BrcGMP on intracellular Ca^{2+} mobilization, it showed little in-

hibitory effect on superoxide production and no effect on myeloperoxidase release in fMLP-activated neutrophils. Thus, the attenuation of intracellular Ca^{2+} mobilization by elevated cGMP level may not affect neutrophil responses.

In conclusion, fMLP-stimulated neutrophil responses may be regulated by cAMP more than cGMP. cAMP and cGMP appear not affect stimulate responses by direct protein kinase C activation. Their regulatory actions on the stimulated neutrophil responses may be not influenced by other activation processes.

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