Biochemical Pharmacology, Vol. 44, No. 6, pp. 1045–1052, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

NEUTROPHIL PRIMING BY GRANULOCYTE COLONY STIMULATING FACTOR AND ITS MODULATION BY PROTEIN KINASE INHIBITORS

MASANOBU TANIMURA, HIROTSUGU KOBUCHI,* TOSHIHIKO UTSUMI,†
TAMOTSU YOSHIOKA,‡ SHINICHI KATAOKA, YUKITOSHI FUJITA and KOZO UTSUMI*§

Departments of Urology and *Medical Biology, Kochi Medical School, Nankoku-shi, Kochi 783;
†Laboratory of Biological Chemistry, Faculty of Agriculture, Yamaguchi University,
Yamaguchi 753; and ‡Center for Adult Diseases, Kurashiki 710, Japan

(*Received* 22 *April* 1992; *accepted* 25 *June* 1992)

Abstract—Upon stimulation by various ligands, freshly isolated human peripheral neutrophils (PMN) respond in a variety of ways, such as superoxide (O_2^-) generation, phagocytosis, enzyme release, migration etc. Chemotactic peptide formylmethionyl-leucyl-phenylalanine (FMLP) and opsonized zymosan activate neutrophils by a receptor-mediated mechanism, while phorbol myristate acetate and dioctanoylglycerol activate the cells by a mechanism involving Ca^{2+}- and phospholipid-dependent protein kinase (PKC). Receptor-mediated but not PKC-mediated O_2^- generation in PMN was enhanced by the priming of recombinant human granulocyte colony stimulating factor (G-CSF). FMLP-dependent luminol chemiluminescence was also enhanced by G-CSF. However, no appreciable enhancement was observed in FMLP-induced intracellular calcium ion concentration ($[Ca^{2+}]_i$). Enhancement of FMLP-induced generation of O_2^- by G-CSF was inhibited by genistein or α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST 638), inhibitors of tyrosine kinase (TK), and was stimulated by staurosporine and 1-(5-isoquinolinesulfonyl)-3-methyl-piperazine (H-7), inhibitors of PKC. The ED_{50} values of genistein and ST 638 for the inhibition of the FMLP-induced O_2^- generation from G-CSF were 0.5 and 5 μM, respectively. In contrast, O_2^- generation by PKC activation without G-CSF priming was inhibited by stauroporine and H-7, but was stimulated by genisten and ST 638. These results suggested that the enhancing effect of G-CSF on receptor-mediated generation of the O_2^- might be regulated by protein kinases, such as TK and PKC, and that the TK inhibitor selectively inhibited the G-CSF-primed receptor-mediated O_2^- generation of neutrophils.

Since peripheral neutrophils of healthy human subjects are not primed, stimulation-dependent responses of these cells are weak. However, when primed by human tumor necrosis factor-α (TNF-α‖) [1], granulocyte–macrophage colony stimulating factor (GM-CSF) [2] or lipopolysaccharide (LPS) [3], they react strongly to various stimuli. Exposure of primed neutrophils to a variety of stimuli triggers the so-called "respiratory burst" in which superoxide radicals generated by neutrophils are converted to more reactive species of active oxygen with microbicidal action [4, 5]. The initial reaction to form superoxide (O_2^-) is the NADPH-dependent one-electron reduction of molecular oxygen by NADPH-oxidase [6]. Since phorbol 12-myristate 13-acetate (PMA) can activate neutrophils and produce O_2^-, Ca^{2+} and phospholipid-dependent protein kinase (PKC) might be involved in stimulation-dependent O_2^- generation [7]. However, an inhibitor of PKC and cyclic nucleotide-dependent protein kinase, H-7, failed to inhibit neutrophil responses induced by formylmethionyl-leucyl-phenylalamine (FMLP) [8]. Thus, the role of PKC in the mechanism of NADPH-oxidase activition remains to be elucidated [9–12]. This raises the question as to whether other protein kinases may also be involved in signal transduction in neutrophils. PKC-dependent and independent activation of NADPH-oxidase in human neutrophils has been described recently [13]. Furthermore, it has been found that tyrosine kinase (TK) is distributed in both cytosolic and membrane fractions of neutrophils, and that the TK inhibitor inhibited particulate but not cytosolic TK activity and the ligand-mediated O_2^- generation of intact human peripheral blood polymorphonuclear leukocytes (PMN) [14]. Similar regulation of tyrosine phosphorylation as an early event in FMLP stimulation was also observed in rabbit peritoneal neutrophils [15]. It has also been reported that GM-CSF primed PMN and increased the tyrosine phosphorylation of cytoplasmic proteins with molecular masses of 118, 92, 78, 54 and 40 kDa [16]. A similar enhancement of tyrosine phosphorylation by GM-CSF and platelet-activating factor was observed by other investigators [17, 18]. Thus, the enzymic

§ Corresponding author. Tel. (81) 888-66-6178; FAX (81) 888-66-6178.

‖ Abbreviations: Cyt. *c*, ferricytochrome *c*; DOG, L-α-1,2 dioctanoyl glycerol; FMLP, formylmethionyl-leucyl-phenylalanine; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte–macrophage colony stimulating factor; H-7, 1-(5-isoquinolinesulfonyl)-3-methyl-piperazine; PMN, human peripheral blood polymorphonuclear leukocytes; KRP, Krebs–Ringer-phosphate; LCL, luminol chemiluminescence; O_2^-, superoxide; OZ, opsonized zymosan; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; TNF-α, human tumor necrosis factor-α; ST 638, α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide; TK, tyrosine kinase.

mechanism for NADPH-oxidase activation is still poorly understood. In a previous paper, we described the two possible pathways for stimulation-coupled O_2^- generation in PMN. One pathway involves TNF-α-primed and receptor-mediated O_2^- generation which is sensitive to TK inhibitors, and the other involves PKC mediated O_2^- generation which is sensitive to PKC inhibitors [19].

Granulocyte colony stimulating factor (G-CSF) is a lineage-specific hematopoietin and is a glycoprotein with 174 (human) or 178 (murine) amino acids. This cytokine is likely to play a role in the regulation of neutrophil production as well as being a regulatory factor controlling the neutrophil response to inflammatory stimuli. G-CSF specifically stimulates colony formation of neutrophilic granulocytes from the bone marrow and stimulates differentiation of myeloblasts [20, 21]. G-CSF also stimulates proliferation of murine and human myeloid leukemia cells, such as NSF-60 [22], AML-193 [23] and OCl/AML 1 cells [24]. Furthermore, G-CSF exhibits other biological activities, such as modulation of neutrophil functions. It has been reported that G-CSF potentiates responses triggered by receptor-mediated agonists [25] and enhances O_2^- release in human granulocytes stimulated by chemotactic peptide [26], and that the priming action of G-CSF is different from that of GM-CSF, quantitatively and qualitatively [27]. Thus, the effect of G-CSF on the responses triggered by receptor-mediated agonists should be studied to understand the mechanism for neutrophil priming and NADPH-oxidase activation.

The present study shows the selective enhancement of ligand-mediated responses of PMN by recombinant human G-CSF. We also describe the modulation of G-CSF-enhanced generation of O_2^- by various protein kinase inhibitors, such as genistein [28], α - cyano - 3 - ethoxy - 4 - hydroxy - 5 - phenylthiomethylcinnamamide (ST 638) [14], 1-(5-isoquinolinesulfonyl)-3-methyl-piperazine (H-7) [8, 29] and staurosporine [30].

MATERIALS AND METHODS

Chemicals. Ferricytochrome *c* (Cyt. *c*), FMLP, PMA, superoxide dismutase, staurosporine and zymosan were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). H-7 was purchased from the Seikagaku Kogyo Co. (Tokyo, Japan) and L-α-1,2 dioctanoyl glycerol (DOG) was obtained from the Funakoshi Co. (Tokyo, Japan). Cyanine dye, 3,3'-dipropylthiadicarbocyanine iodide, was donated by the Kankoshikiso Research Institute (Okayama, Japan). G-CSF was donated by the Chugai Pharmaceutical Co. (Tokyo, Japan). TNF-α was donated by the Hayashibara Bioscience Research Institute (Okayama, Japan) and ST 638 was kindly provided by Dr Tadayoshi Shiraishi, Biochemical Research Laboratory, Kanegafuchi Chemical Industry Co. (Hyogo, Japan). Fura-2 acetomethoxy pentaester, purchased from Dojindo Laboratories (Kumamoto, Japan), was used in solution with dimethylsulfoxide at dimethylsulfoxide concentrations less than 30 μM. All other chemicals used were of analytical grade and were obtained from the Nakarai Tesque Co. (Kyoto, Japan). Luminol was used as a triethylamine solution [5].

Stimulation of neutrophils. FMLP (1.25×10^{-8} M) and opsonized zymosan (OZ, 200–400 μg/mL) were used for the receptor-mediated activation of neutrophils. PMA ($1–3 \times 10^{-9}$ M) and DOG ($0.3–1 \times 10^{-6}$ M) were used as activators of PKC.

Preparation of human peripheral neutrophils. PMN were isolated from the venous blood of healthy human subjects by Ficoll-Hypaque density gradient centrifugation. Isolated neutrophils were washed twice with Krebs–Ringer-phosphate buffer solution (KRP, pH 7.4) [19]. Cells were counted and resuspended in KRP at a concentration of 1×10^8 cells/mL. Cell viability was tested by trypan blue dye exclusion test.

Measurement of O_2^- generation. O_2^- generation was assayed by reduction of Cyt. *c* using a dual beam spectrophotometer (Shimadzu UV 300) equipped with a water-jacketed cell holder and magnetic stirrer [5]. The reaction mixture contained a final volume of 2 mL KRP, 1 mM $CaCl_2$ 20 μM Cyt. *c*, 10 mM glucose and $0.2–1 \times 10^6$ cells/mL. The reaction was initiated by adding a ligand in the presence or absence of various concentrations of G-CSF, and the change in absorbance at 550–540 nm ($A_{550-540}$) was monitored at 37°. The rate of O_2^- generation was calculated from the superoxide dismutase-inhibitable Cyt. *c* reduction using the extinction coefficiency of 21.0 mM^{-1} [31].

Measurement of luminol chemiluminescence (LCL). LCL experiments were performed using an ATP photometer (All Co. Type 401) or calcium analyser (Jasco CAF 100, aequorin chemiluminescence mode) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer [5]. The reaction mixture contained, in a final volume of 1 mL KRP, $1–10 \times 10^{-5}$ M luminol, $0.2–1 \times 10^6$ cells and other additions. The reaction was initiated by adding a test ligand in the presence or absence of various concentrations of G-CSF, and light emission was recorded for 15 min at 37°. LCL was determined by measuring its peak height (counts per minute) or by calculating the area under the LCL light curve (intregral LCL).

Measurement of intracellular Ca^{2+} concentration. The intracellular calcium ion concentration ($[Ca^{2+}]_i$) was calculated from changes in the fluorescence intensity of Fura-2-loaded PMN (2×10^6 cells/mL). The loading of Fura-2 and the calibration of Fura-2-Ca^{2+} as a function of $[Ca^{2+}]_i$ were essentially the same as described by Ozaki and Kume [32]. The fluorescence intensity of Fura-2-loaded PMN was measured with a calcium analyser (Jasco CAF 100) at 37° in the presence or absence of G-CSF.

RESULTS AND DISCUSSION

Enhancement of FMLP-dependent O_2^- generation in PMN by G-CSF

Without treatment with G-CSF, the degree of O_2^- generation of FMLP-stimulated PMN was very low. As reported previously [25], PMN were primed by G-CSF, and FMLP-induced O_2^- generation was increased significantly by incubation with 50 ng/mL

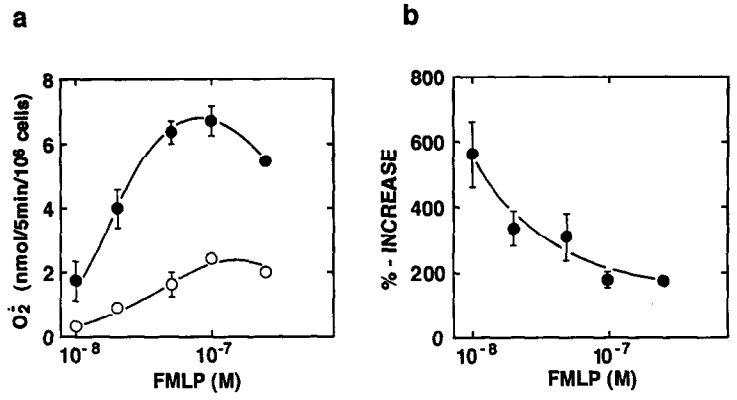

Fig. 1. Enhancement of FMLP-induced O_2^- generation by G-CSF and its dependence on the FMLP concentration. PMN (1×10^6 cells/mL) were incubated at 37° in 2 mL of KRP containing 1 mM $CaCl_2$, 10 mM glucose, 20 μM Cyt. *c*, in the presence or absence of G-CSF and various concentrations of FMLP. The change in absorbance at 550–540 nm ($A_{550-540}$) was monitored and O_2^- generation was calculated from superoxide dismutase-inhibitable Cyt. *c* reduction. (a) O_2^- generation (nmol/5 min/10^6 cells) in FMLP-stimulated PMN after preincubation with or without 50 ng/mL G-CSF for 10 min (●) in the presence of G-CSF (○) in the absence of G-CSF. (b) Stimulation of O_2^- generation by G-CSF in the response to various concentrations of FMLP was expressed as percentage increase by 50 ng/mL G-CSF. Each data point is the mean ± SE of triplicate incubations.

G-CSF. The enhanced response of FMLP-stimulated cells was 5-fold higher than that of controls cells (Fig. 1a and b). However, the rates of enhancement differed with different concentrations of FMLP; higher responses occurred with lower FMLP concentrations than with higher concentrations (Fig. 1b). In contrast, PMA failed to enhance O_2^- generation of G-CSF-primed PMN (data not shown) as observed by Yuo *et al.* [26]. Similar results were also observed with PMN which were primed by TNF-α [33, 34].

Enhancement of stimulus-dependent O_2^- generation by G-CSF

Stimulus-dependent O_2^- generation was enhanced by G-CSF concentration dependently (Fig. 2a and b). The half maximum concentration for priming was about 50 ng/mL. In contrast, the rate of O_2^- generation induced by PKC activators (PMA or DOG) was very high without priming by G-CSF: no stimulatory action of G-CSF was observed even at a concentration of 100 ng/mL (Fig. 2b). These results suggested that ligand-mediated O_2^- generation of neutrophils was highly sensitive to priming by G-CSF. The priming action of G-CSF also depended on the preincubation time before treatment with FMLP (Fig. 3). Longer than 15 min was required to obtain maximal stimulation and half-maximal stimulation was obtained at about 8 min. These profiles of G-CSF action were quite similar to those of TNF-α [19].

Effects of G-CSF on stimulus-dependent LCL

Reactive oxygen species released by neutrophils involve O_2^-, H_2O_2, $\cdot OH$, $^1O_2$ and OCl^- [5, 34, 35]. O_2^- generation is the initial step to produce various active oxygen species. It is believed that LCL reflects mainly OCl^- and $\cdot OH$. The FMLP-induced LCL response of PMN was very small and no significant effect was observed with 50 ng/mL G-CSF. However, the FMLP-induced LCL response was strongly increased by G-CSF in a concentration-dependent manner (Fig. 4a). Similar enhancement of LCL by G-CSF was also seen with OZ stimulation (data not shown). In contrast, its effect was very small with PMA-induced stimulation. Similar effects of G-CSF were also observed with a DOG-induced LCL response (data not shown). Thus, the degree of response to each stimulus differed significantly and the enhancing effect was greater with receptor-mediated ligand than that observed with PKC activator (Fig. 4b). In this case, the order of LCL enhancement was similar to that of O_2^- generation. These results suggest that the enhancement by G-CSF of the receptor-mediated LCL response of PMN depends on the oxygen species derived from enhanced O_2^- production.

Effect of G-CSF on the $[Ca^{2+}]_i$ of PMN

$[Ca^{2+}]_i$ plays an important role in cell signal transduction. FMLP, but not PMA, transiently increased neutrophil $[Ca^{2+}]_i$ [32] as determined by the Fura-2 method [36]. When calcium ions were added exogenously, the $[Ca^{2+}]_i$ increased transiently (Fig. 5) and reached a stationary phase. This change was attributed to an influx of external Ca^{2+}, and was not affected by preincubating cells with G-CSF. The $[Ca^{2+}]_i$ was changed further by adding FMLP. However, no significant change in the $[Ca^{2+}]_i$ or in FMLP-induced responses was found even after 30 min preincubation with G-CSF.

Effects of protein kinase inhibitors on priming by G-CSF

We previously reported strong inhibition of ligand-dependent O_2^- generation in TNF-α-primed PMN

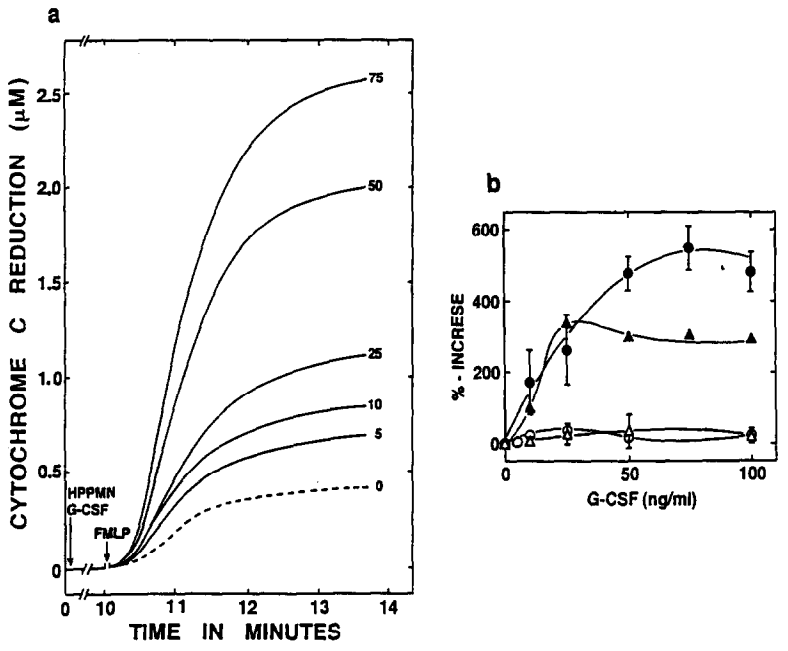

Fig. 2. Enhancement of stimulus-dependent O_2^- generation by G-CSF and its dependence on the G-CSF concentration. Experimental conditions were the same as described in Fig. 1. Concentrations of FMLP, OZ, PMA and DOG were 1.25×10^{-8} M, 200 μg/mL, 1×10^{-9} M and 0.7×10^{-6} M, respectively. Values of G-CSF dose–response for the stimulus-dependent O_2^- generation are expressed as the means ± SE of 3–5 separate experiments, and are presented as percentages of control. Control O_2^- production (nmol/5 min/10^6 cells) of PMN by the treatment with FMLP, OZ, PMA and DOG was 0.42 ± 0.7, 0.11 ± 0.03, 11.3 ± 3.25 and 3.58 ± 1.26, respectively. (a) Actual traces of O_2^- generation in the presence of various concentrations of added G-CSF (ng/mL). (b) % increase by G-CSF; (●) FMLP, (▲) OZ, (○) PMA, (△) DOG.

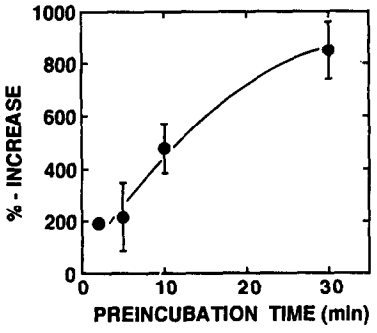

Fig. 3. Effect of preincubation time with G-CSF on the FMLP-induced O_2^- generation of PMN. Experimental conditions were the same as described in Fig. 1. Concentrations of FMLP and G-CSF were 1.25×10^{-8} M and 50 ng/mL, respectively. Each data point is the mean ± SE of triplicate incubations.

by TK inhibitors [19]. A similar inhibitory effect of TK inhibitors on FMLP-dependent O_2^- generation by G-CSF-primed PMN was observed. Figure 6a shows the FMLP-induced O_2^- generation by G-CSF-primed PMN and its inhibition by genistein, an inhibitor of TK [28]. The inhibitory action of genistein depended on its concentration (Fig. 6a and b), and the ED_{50} was 0.5 μM. The concentration-dependent inhibition by genistein occurred similarly to that of FMLP-induced O_2^- generation by TNF-α-primed PMN [19]. Furthermore, similar inhibition was induced by another TK inhibitor, ST 638 [14] (Fig. 6b). In contrast, FMLP-induced O_2^- generation by G-CSF-primed PMN was increased further by treating with low concentrations of H-7 and staurosporine, protein kinase C inhibitors [29, 30] (Fig. 6a and b), as was observed for FMLP-induced O_2^- generation by TNF-α-primed PMN [19]. Maximum stimulation was observed at 25 nM staurosporine and 25 μM H-7. PKC inhibitors at these concentrations inhibited the O_2^- generation induced by PMA. These results suggested that TK is involved in FMLP-mediated O_2^- generation by PMN which were pretreated with G-CSF and that PKC and TK regulate the activation mechanism of NADPH-oxidase.

Effect of protein kinase inhibitors on PMA- and DOG-induced O_2^- generation

Without priming of PMN, the rate of O_2^- generation induced by 1×10^{-9} M PMA or 7×10^{-7} M DOG was very high, and both PMA- and DOG-induced O_2^- generation was inhibited by 10 nM staurosporine and 50 μM H-7 (Fig. 7). In contrast, the PMA- or DOG-induced O_2^- generation

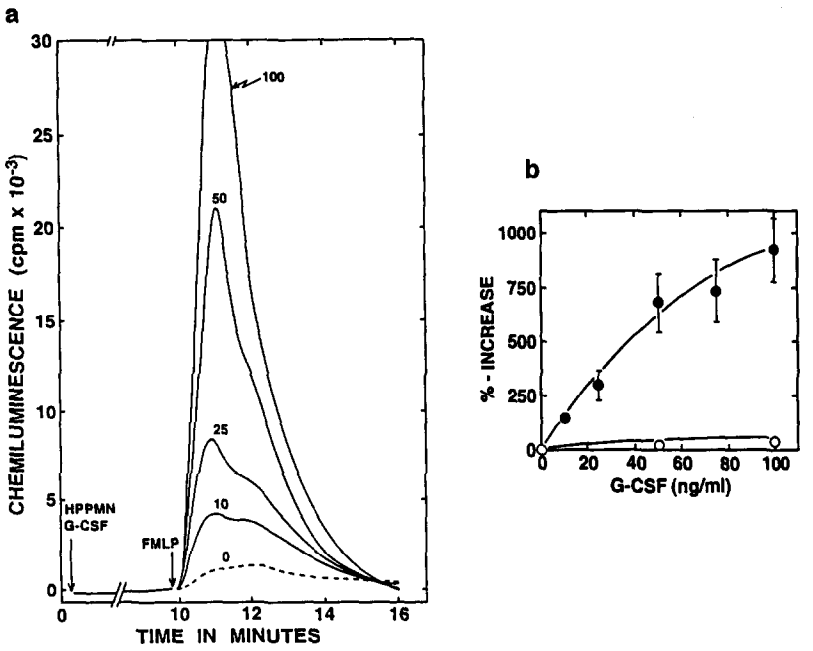

Fig. 4. Effect of G-CSF on stimulation-dependent LCL response in PMN. LCL was measured in the same medium as that described in the legend for Fig. 1 except that 100 μM luminol was added instead of Cyt. *c*. LCL was monitored by a calcium analyser. Concentrations of FMLP and PMA were 1.25×10^{-8} and 1×10^{-9} M, respectively. (a) FMLP-induced LCL response after 10 min preincubation with various concentrations of G-CSF; concentrations of added G-CSF (ng/mL) are indicated. (b) % increase in stimulation-dependent LCL response with various concentrations of G-CSF; (●) 1.25×10^{-8} M FMLP-induced LCL, (○) 1×10^{-9} M PMA-induced LCL; each data point is the mean ± SE of triplicate incubations.

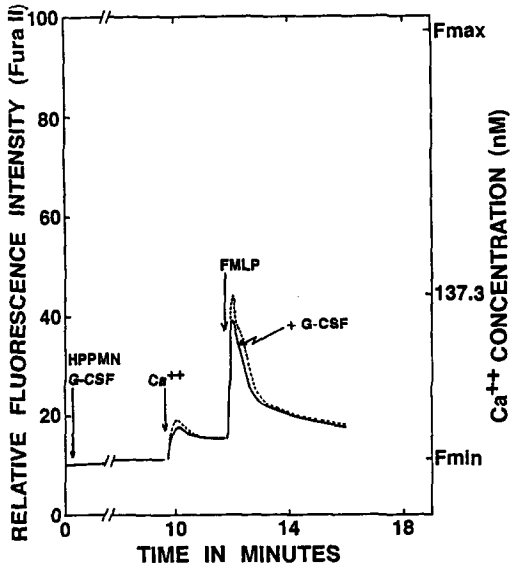

Fig. 5. Effect of G-CSF on the stimulation-coupled changes in $[Ca^{2+}]_i$ of PMN. Fura-2-loaded PMN (2×10^6 cells/mL) were incubated in KRP (pH 7.4) containing 10 mM glucose. Changes in fluorescence at 37° were measured by a calcium analyser. The concentrations of calcium, FLMP, Triton X-100, Tris and EGTA were 1 mM, 2.5×10^{-8} M, 0.1%, 5 mM and 5 mM, respectively. Solid line represents the trace after preincubation with 50 ng/mL of G-CSF; broken line represent the trace without G-CSF.

was stimulated by 25 μM genistein and 20 μM ST 638. These concentrations of TK inhibitors can inhibit FMLP-induced O_2^- generation by G-CSF-primed PMN. These data suggest that PKC is involved in PMA- or DOG-mediated activation of NADPH-oxidase and that the activation mechanism of the oxidase is regulated by both PKC and TK.

The present study, confirmed our previous observations [19, 35] and those of Yuo and coworkers [25, 26] that G-CSF enhanced the receptor-mediated stimulation of O_2^- generation without affecting the increase in $[Ca^{2+}]_i$. Furthermore, we found that G-CSF enhanced the receptor-mediated LCL response in PMN. The enhancement by G-CSF, however, was not observed with the responses mediated by PKC activators, such as PMA and DOG. These findings suggest that stimulus-coupled responses of PMN are enhanced by G-CSF through signal transduction mechanisms other than changes in $[Ca^{2+}]_i$ and activation of PKC as reported by Yuo *et al.* [26]. The priming action of G-CSF was quite similar to that of TNF-α [19, 34, 37].

From studies with neutrophils from patients with chronic granulomatous disease, a mechanism for NADPH-oxidase activation by PMA has been postulated to involve a PKC-dependent pathway [10, 11, 38]. However, some groups have argued against this hypothesis [39–41], and Watson *et al.* [42] have proposed two pathways for the activation of NADPH-oxidase: one involves PKC-dependent phosphorylation and the other is PKC independent. Thus, the role of PKC in NADPH-oxidase activation

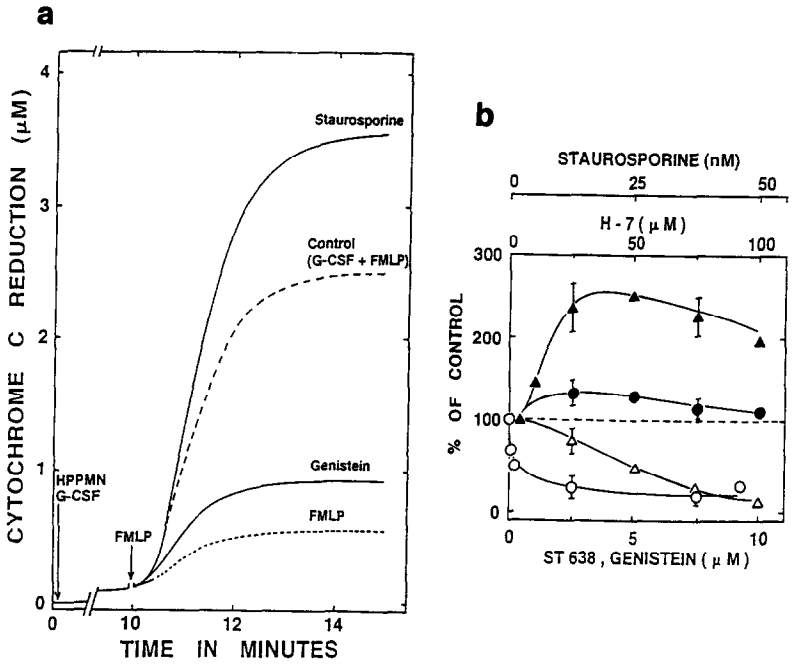

Fig. 6. Effects of protein kinase inhibitors on the G-CSF-primed FMLP-induced O_2^- generation of PMN. Experimental conditions were the same as described in Fig. 1. PMN (1×10^6 cells/mL) were preincubated with 50 ng/mL G-CSF for 10 min, and stimulated with 1.25×10^{-8} M FMLP in the presence of 5 μM staurosporine and 18.5 μM genistein at 37°. Various inhibitors were added with G-CSF. (a) O_2^- generation monitored by Cyt. *c* reduction. (b) Dose–response curves of various inhibitors for the inhibition of G-CSF-primed FMLP-induced O_2^- generation; each point is the mean ± SE of triplicate incubations; (●) H-7, (▲) staurosporine, (○) genistein, (△) ST 638.

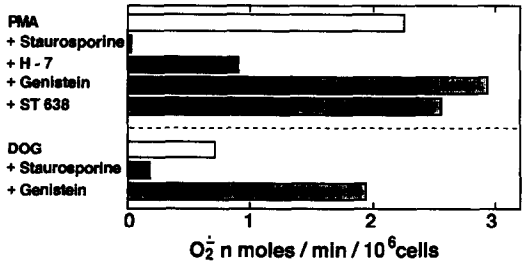

Fig. 7. Effect of protein kinase inhibitors on PMA- and DOG-induced O_2^- generation. PMN (1×10^6 cells/mL) were incubated in the medium of KRP containing 1 mM $CaCl_2$, 10 mM glucose, 20 μM Cyt. *c* in the presence or absence of 10 nM staurosporine, 50 μM H-7, 25 μM genistein and 10 μM ST 638 at 37°. The concentrations of PMA and DOG were 1×10^{-9} and 7×10^{-7} M, respectively. Data are means ± SE of triplicate experiments.

remains to be elucidated. In this experiment, we found that when suboptimal concentrations of stimuli were used the PKC inhibitors inhibited the PMA- or DOG-induced O_2^- generation of PMN whereas the TK inhibitors stimulated it. In contrast, FMLP-induced O_2^- generation of G-CSF-treated PMN was stimulated by low concentrations of H-7 and staurosporine, while it was inhibited by genistein and ST 638 as was observed for TNF-α-induced priming of PMN [19]. A variety of evidence has accumulated concerning the tyrosyl phosphorylation of neutrophils by treatment with priming agents [15–18]. In a preliminary experiment, we observed a similar activation of tyrosyl phosphorylation of PMN on treatment with G-CSF, indicating that G-CSF may activate TK activity through a unique mechanism. This, taken together with the present findings, suggests that both PKC and TK are involved in the mechanism for FMLP-induced O_2^- generation by G-CSF-primed PMN as was the case with FMLP-induced O_2^- generation by TNF-α-primed PMN [19].

Acknowledgements—This work was supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan. A part of this work was also supported by a fund of Japan Keirine Association. We would like to thank Mrs Yoko Akimaru for her excellent technical help.

REFERENCES

1. Shalaby MR, Aggarwal BB, Rinderknecht E, Svedersky LP, Finkle BS and Palladino MA, Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumour necrosis factors. *J Immunol* **135**: 2069–2073, 1983.
2. Wirthmueller U, De Weck AL and Dahinden CA, Platelet-activating factor production in human neutrophils by sequential stimulation with granulocyte-macrophage colony-stimulating factor and the chemotactic factors C5A or formyl-methionyl-leucyl-phenyalanine. *J Immunol* **142**: 3213–3218, 1989.

3. Guthrie LA, McPhail LC, Henson PM and Johnston RB, Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopoly-saccharide. Evidence for increased activity of the superoxide-producing enzyme. *J Exp Med* **160**: 1656–1671, 1984.
4. Babior BM, Kipnes RS and Curnette JT, Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest* **52**: 741–744, 1973.
5. Takahashi R, Edashige K, Sato EF, Inoue M, Matsuno T and Utsumi K, Luminol chemiluminescence and active oxygen generation by activated neutrophils. *Arch Biochem Biophys* **285**: 325–330, 1991.
6. Rossi F, The O_2^--forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and functions. *Biochim Biophys Acta* **853**: 65–89, 1986.
7. Nishizuka Y, The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308**: 693–698, 1984.
8. Sha'afi RI, Molski TF, Gomez-Cambronero J and Huang CK, Dissociation of the 47-kilodalton protein phosphorylation from degranulation and superoxide production in neutrophils. *J Leukocyte Biol* **43**: 18–27, 1988.
9. Heyworth PG, Curnutte JT, Nauseef WM, Volpp BD, Pearson DW, Rosen H and Clark RA, Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J Clin Invest* **87**: 352–356, 1991.
10. Rotrosen D and Leto TL, Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. *J Biol Chem* **265**: 19910–19915, 1990.
11. Nauseef WM, Volpp BD, McCormick S, Leidal KG and Clark RA, Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J Biol Chem* **266**: 5911–5917, 1991.
12. Sharma P, Evans AT, Parker PJ and Evans FJ, NADPH-oxidase activation by protein kinase C-isotopes. *Biochem Biophys Res Commun* **177**: 1033–1040, 1991.
13. Watson F, Robinson J and Edwards SW, Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils. *J Biol Chem* **266**: 7432–7439, 1991.
14. Berkow RL, Dodson EW and Kraft AS, Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role in neutrophil activation. *Biochim Biophys Acta* **997**: 292–301, 1989.
15. Huang CK, Bonak V, Laramee GR and Casnellie JE, Protein tyrosine phosphorylation in rabbit peritoneal neutrophils. *Biochem J* **269**: 431–436, 1990.
16. Gomez-Cambronero J, Yamazaki M, Metwally F, Molksi TFP, Bonak VA, Huang C, Becker EL and Shaafi RI, Granylocyte–macrophage colony-stimulating factor and human neutrophils. Role of guanine nucleotide regulatory protien. *Proc Natl Acad Sci USA* **86**: 3659–3573, 1989.
17. McColl SR, DiPersio JF, Caon AC, Ho P and Naccache PH, Involvement of tyrosine kinase in the activation of human peripheral blood neutrophils by granulocyte–macrophage colony-stimulating factor. *Blood* **78**: 1842–1852, 1991.
18. Gomez-Cambronero J, Wang E, Johnson G, Huang C and Sha'afi RI, Platelet-activating factor induces tyrosine phosphorylation in human neutrophils. *J Biol Chem* **266**: 6240–6245, 1991.
19. Utsumi T, Klostergaard J, Akimaru K, Edashige K, Sato EF and Utsumi K, Modulation of TNF-α-priming and stimulation dependent superoxide generation in human neutrophils by protein kinase inhibitors. *Arch Biochem Biophys* **294**: 271–278, 1992.
20. Nagata S, Tsuchiya M, Asano S, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H and Yamazaki T, The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor. *EMBO J* **5**: 575–581, 1986.
21. Tuchiya M Asano S, Kaziro Y and Nagata S, Isolation and characterization of the cDNA for murine granulocyte colony-stimulating factor. *Proc Natl Acad Sci USA* **83**: 7633–7637, 1986.
22. Weinstein Y, Ihle JN, Lavu S and Reddy EP, Truncation of the c-myb gene by a retroviral integration in an interleukin 3-dependent myeloid leukemia cell line. *Proc Natl Acad Sci USA* **83**: 5010–5014, 1986.
23. Dantoli D, Yang YC, Clark SC, Kreider BL, Caracciolo D and Rovera G, Synergistic and antagonistic effects of recombinant human interleukin (IL) 3, IL-1 alpha, granulocyte and macrophage colony-stimulating factors (G-CSF and M-CSF) on the growth of GM-CSF-dependent leukemic cell lines. *J Immunol* **139**: 3348–3354, 1987.
24. Nara N, Suzuki T, Nagata K, Tohda S, Yamashita Y, Nakamura Y, Imai Y, Morio T and Minamihisamatsu M, Granulocyte colony-stimulating factor-dependent growth of an acute myeloblastic leukemia cell line. *Jpn J Cancer Res* **81**: 625–631, 1990.
25. Kitagawa S, Yuo A, Souza LM, Saito M, Miura Y and Takaku F, Recombinant human granulocyte colony-stimulating factor enhances superoxide release in human granulocytes stimulated by the chemotactic peptide. *Biochem Biophys Res Commun* **144**: 1143–1146, 1987.
26. Yuo A, Kitagawa S, Ohsaka A, Ohta M, Miyazono K, Okabe T, Urabe A, Saito M and Takaku F, Recombinent human granulocyte colony-stimulating factor as an activator of human granylocytes: potentiation of responses triggered by receptor-mediated agonists and stimulation of C3bi receptor expression and adherence. *Blood* **74**: 2144–2149, 1989.
27. Yuo A, Kitagawa S, Ohsaka A, Saito M and Takaku F, Stimulation and priming of human neutrophils by granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor: qualitative and quantitative differences. *Biochem Biophys Res Commun* **171**: 491–497, 1990.
28. Akiyama T, Ishida J, Nakagawa S, Ogawa H, Watanabe S, Ito N, Shibuya M and Fukami Y, Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* **262**: 5592–5595, 1987.
29. Kawamoto S and Hidaka H, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. *Biochem Biophys Res Commun* **125**: 258–264, 1984.
30. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F, Stauroporine, a potent inhibitor of phospholipid/Ca^{++} dependent protien kinase. *Biochem Biophys Res Commun* **135**: 397–402, 1986.
31. Gelder VBF and Slater EC, The extinction coefficient of cytochrome *c*. *Biochim Biophys Acta* **58**: 593–595, 1962.
32. Ozaki Y and Kume S, Functional responses of aequorin-loaded human neutrophils. Comparison with Fura-2 loaded cells. *Biochim Biophys Acta* **972**: 113–119, 1988.
33. Atkinson YH, Marasco WA, Lopez AF and Vadas MA, Recombinant human tumor necrosis factor-a. Regulation of *N*-formylmethionylleucylphenylalanine receptor affinity and function of human neutrophils. *J Clin Invest* **81**: 759–765, 1988.
34. Utsumi T, Klostergaard J, Akimaru K, Sato EF, Yoshioka T and Utsumi K, Effect of tumor necrosis factor-α on the stimulus-coupled responses of neu-

trophils and their modulation by various inhibitors. Physiol Chem Phys Med NMR **24**: 77–88, 1992.
35. Allen RC, Phagocytic leukocyte oxygenation activities and chemiluminescence. A kinetic approach to analysis. *Methods Enzymol* **133**: 449–493, 1986.
36. Cobbold PH and Rink TJ, Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem J* **248**: 313–328, 1987.
37. Yuo A, Kitagawa S, Suzuki I, Urabe A, Okabe T, Saito M and Takaku F, Tumor necrosis factor as an activator of human granulocytes. Potentiation of the metabolisms triggered by the Ca^{2+}-mobilizing agonists. *J Immunol* **142**: 1678–1684, 1989.
38. Caldwell SE, McCall CE, Hendricks CL, Leone PA, Bass DA and McPhaill LC, Coregulation of NADPH oxidase activation and phosphorylation of a 48-kDa protein(s) by a cytosolic factor defective in autosomal recessive chronic granulomatous disease. *J Clin Invest* **81**:1485–1496, 1986.
39. Badwey JA, Horn W, Heyworth PG, Robinson JM and Karnovsky ML, Paradoxical effects of retinal in neutrophil stimulation. *J Biol Chem* **264**: 14947–14953, 1989.
40. Badwey JA, Robinson JM, Heyworth PG and Curnutte JT, 1,2-dioctanoyl-*sn*-glycerol can stimulate neutrophils by different mechanisms. Evidence for a pathway that does not involve phosphorylation of the 47-kDa protein. *J Biol Chem* **264**: 20676–20682, 1989.
41. Tamura M, Tamura T, Tyagi SR and Lambeth JD, The superoxide-generating respiratory burst oxidase of human neutrophil plasma membrane. Phosphatidylserine as an effector of the activated enzyme. *J Biol Chem* **263**: 17621–17626, 1988.
42. Watson F, Robinson J and Edwards SW, Protein kinase C-dependent and -independent activation of the NADPH-oxidase of human neutrophils. *J Biol Chem* **266**: 7432–7439, 1991.