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ARTICLES

Granulocyte Colony-Stimulating Factor-Induced Activation of Protein Kinase-C in Myeloid Cells

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Abstract Granulocyte colony stimulating factor (G-CSF) regulates survival, proliferation, differentiation, and activation of myeloid cells. It binds to a high affinity receptor (G-CSF-R) expressed on myeloid cells, for which the signal transduction mechanisms other than protein tyrosine kinase (PTK) activation have not been completely identified. We explored the potential involvement of protein kinase-C (PKC) in G-CSF-R signal transduction. In this report, we provide direct evidence of PKC activation by G-CSF-R. G-CSF treatment of peripheral blood neutrophils, granulocytic cell lines (HL-60, NFS-60, KG-1), and monocytic cell lines (WEHI-3B,U-937) resulted in PKC activation. Chelerythrine chloride and HA-100, an isoquinolinesulfonamide derivative, the specific inhibitors of PKC, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (BAPTA), a chelator of intracellular calcium, and 3,4,5-trimethoxybenzoic acid 8-(diethyl-amino)-octyl ester (TMB-8), an inhibitor of intracellular calcium release, blocked G-CSF-induced PKC activation in HL-60 cells, and reduced CD11b upregulation in neutrophils, but did not affect ligand-binding or down-modulation of G-CSF-R. Methyl 2,5-dihydroxycinnamate (MDHC), a potent inhibitor of protein tyrosine kinases (PTK), also inhibited PKC activation in response to G-CSF treatment, suggesting that PKC activation may occur downstream of PTK activation. Our results demonstrate the involvement of PKC in G-CSF-R signal transduction, and suggest a common signaling pathway in myeloid cells of granulocytic and monocytic lineages. J. Cell. Biochem. 66:286–296, 1997.

Key words: cytokine; signaling; neutrophil; protein kinase C; myeloid; colony-stimulating; receptor

Granulocyte colony-stimulating factor (G-CSF) supports survival, proliferation, and differentiation of myeloid progenitors, and enhances effector functions of neutrophils [Nicola, 1987; Clark and Kamen, 1987]. The G-CSF receptor (G-CSF-R) is expressed on neutrophils, monocytes, bone marrow cells, endothelial cells, placenta, trophoblasts, myeloid leukemia cells, and small cell lung cancer cell lines [Nicola and Metcalf, 1984; Begley et al., 1988]. It lacks an intrinsic tyrosine kinase activity, but upon ligand-interaction, triggers activation of JAK and src-family tyrosine kinases, MAP kinase, and STAT transcription factors [Corey et al., 1994; Nicholson et al., 1994; Matsuda and Hirano, 1994; Tian et al., 1994; Avalos, 1996; Deshpande et al., 1997]. While G-CSF-induced tyrosine kinase activation is well documented, little is described about other pathway(s) of G-CSF-R signal transduction. We explored the possibility of protein kinase-C (PKC) regulation as an additional mechanism of G-CSF-R signal transduction.

Protein kinase C (PKC) regulates a number of hormone- and cytokine-induced growth and differentiation responses in many cell types, including immune and inflammatory cells [Nishizuka, 1986; Berridge, 1993]. PKC is expressed in mammalian systems as a family of diverse serine-threonine kinases, consisting of at least 11 isoforms differing in both substrate specificity and Ca^{2+} dependence. They are classified as conventional (α, $\beta1$, $\beta2$, γ), novel (δ, ϵ, η, θ, μ), and atypical (ζ, λ). Several of these isoforms are differentially expressed in hematopoietic cells, and their activation is postulated

Abbreviations: G-CSF, granulocyte colony-stimulating factor; G-CSF-R, granulocyte colony-stimulating factor receptor; PTK, protein tyrosine kinase; PKC, protein kinase-C; BAPTA, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethyl-amino)-octyl ester; MDHC, methyl 2,5-dihydroxycinnamate; PMA, phorbol 12-myristate 13-acetate.

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to account for the divergent actions of different agonists [Nishizuka, 1988; Mischak et al., 1991; Ogita et al., 1992].

To investigate whether G-CSF-R utilizes the PKC pathway for signal transduction, we measured the activity of PKC purified from G-CSF-treated myeloid cell lines and peripheral blood neutrophils. Our results demonstrate that G-CSF induces PKC activation in these cells.

MATERIALS AND METHODS

Cells and Reagents

HL-60 (human promyelocytic leukemia), KG-1 (human acute myelogenous leukemia), U-937 (human histiocytic lymphoma), and WEHI-3B (mouse myelomonocytic leukemia) cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO-BRL) containing 3 g/L $NaHCO_3$, 10% fetal calf serum (Hyclone) and 0.001% α-monothioglycerol. NFS-60 (mouse myeloid leukemia) cells were cultured in the same medium containing rhGM-CSF (2 ng/ml).

Since HL-60 cells showed the highest level of PKC activity, and expressed easily detectable numbers of G-CSF-R, these cells were used for PKC inhibition and receptor binding assays (below). Because NFS-60 cells, but not other cell lines used in the study, proliferate in response to G-CSF, they were used for the thymidine uptake assay. As peripheral blood neutrophils have the highest surface expression of CD11b among the cells in this study, they were used for CD11b regulation assays.

Escherichia coli–derived rhG-CSF and GM-CSF were obtained from Amgen Inc. (Thousand Oaks, CA). BAPTA, TMB-8, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Chelerythrine chloride, HA-100, and MDHC were obtained from LC Laboratories (Woburn, MA). $Na^{125}I$, [^{3}H]thymidine, and [γ^{32}P]ATP, were purchased from New England Nuclear (Boston, MA).

Neutrophil Isolation

Peripheral blood neutrophils from normal, healthy human donors were isolated using the Mono-Poly Resolving Medium (ICN Biochemicals). Fresh, heparinized peripheral blood (3.5 ml) was layered on to 3 ml of Mono-Poly Resolving Medium and centrifuged at 400*g* for 30 min at room temperature in a swing-out-rotor centrifuge. Neutrophils were recovered, washed twice with PBS, and used immediately. The neutrophil population was >97% pure, as evaluated by Giemsa staining and FACS analysis, and 100% viable.

Assay for Protein Kinase C

Protein kinase C activity in myeloid cells was determined using the synthetic peptide substrate, Ac-MBP (4–14) (GIBCO-BRL), the sequence of which was identical to N-terminal residues 4 to 14 in myelin basic protein. Cells (10^6/ml) were treated with 5 nM rhG-CSF for 0–4 h at 37°C, and lysed in 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 10 mM β-mercaptoethanol, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. Cell lysates were incubated on ice for 30 min, and centrifuged at 13,600*g* for 2 min to remove cellular debris. DEAE cellulose (Whatman DE52) was suspended in 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol (Buffer B) (0.5 g DE52/ml buffer B). One milliliter of DE52 slurry was poured into chromatography columns (Bio-Rad, Richmond, CA), and equilibrated with buffer B. Cell-extracts were loaded on the column, and columns were washed with 5 ml buffer B. PKC was then eluted with 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, and 0.2 M NaCl. Aliquots of eluted PKC were pre-incubated with 20 μM PKC inhibitor peptide (PKC 19-36) or lipid preparation (10 μM PMA, 280 μg/ml phosphatidyl serine, Triton X-100 mixed micelles) (GIBCO-BRL) at room temperature for 20 min. Reaction mixtures were further incubated at 30°C for 5 min with 250 μM Ac-MBP and 5 μCi/ml [γ^{32}P]ATP, and 25 μl aliquots were spotted on to phosphocellulose paper-discs. Filters were washed with 1% phosphoric acid, and counted for incorporated radioactivity. PKC activity was represented as pmoles of [γ^{32}P]ATP transferred to Ac-MBP per minute by PKC purified from a million cells. PKC isolated from cells treated with 10 μM PMA were used as positive standards.

PKC and PTK Inhibitors

PKC activity was also measured in cells treated with PKC or PTK inhibitors. Cells were treated with chelerythrine chloride (10 μM), HA-100 (20 μM), BAPTA (10 μM), TMB-8 (50 μM) or MDHC (100 μg/ml) at 37°C for 30

min, followed by G-CSF (5 nM) for 30 min, and PKC activity measured as described.

Iodination of rhG-CSF

E. coli–derived rhG-CSF was iodinated by the chloramine T method, as described previously [Shieh et al., 1991]. Briefly, 35 µl of a reaction mixture containing 0.5 µg G-CSF, 10% DMSO, 0.02% polyethylene glycol, 6 nmol chloramine, 1 mCi carrier-free $Na^{125}I$, and 0.25 M sodium phosphate buffer, pH 6.8, was incubated on ice for 5 min. Thereafter, 6 µl of 1.1 nmol/µl cysteine and 10 µl of 0.2 M KI were added, and the reaction mixture remained on ice for 10 min. The reaction mixture was desalted by passage over a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ), which was conditioned with 5 ml of 10 mg/ml BSA and 20 mM Tris-HCl, pH 6.8. The crude ^{125}I-G-CSF was further fractionated on a Sephacryl S-200 column, with a buffer containing 0.5 M NaCl, 0.02% NaN_3, 0.1 mg/ml BSA, and 20 mM Tris-HCl, pH 8.0. The biological activity of Sephacryl S-200-purified ^{125}I-G-CSF was measured by its potential to support proliferation of NFS-60 cells in [^{3}H]thymidine uptake assay described below.

G-CSF Receptor Binding Assay

^{125}I-G-CSF binding assay was performed as described previously, with minor modifications [Shieh et al., 1991]. Briefly, cells were washed twice with cold PBS, and re-suspended in RPMI 1640 medium (without sodium bicarbonate), 2 mg/ml BSA, 0.02% NaN_3, 20 mM HEPES, pH 7.3 at a density of 10^7 cells/ml. One hundred microliters (10^6 cells) was dispensed per well in a 96-well cell-culture plate (Falcon). To determine the non-specific binding of ^{125}I-G-CSF to cells, 10 µl of unlabeled G-CSF (10 ng) was added per well, and plates were incubated at 4°C for 30 min prior to addition of 10 µl ^{125}I-G-CSF (approximately 50,000 cpm) per well. To measure the total binding of ^{125}I-G-CSF to the receptors, only radiolabeled G-CSF was added to cell suspension. Cells were incubated overnight on a rocking platform at 4°C. Cell-suspensions were then layered on to 200 µl cold FCS in 400-µl centrifuge tubes and centrifuged at 400*g* for 5 min. The bottom-ends of the tubes containing the cell-pellets were excised, and the amount of radioactivity in pellets was counted in a Cobra Auto-Gamma-counter (Packard, Sterling, VA). Specific binding activity was calculated by subtracting the non-specific binding (<15% of total binding) from the total binding of ^{125}I-G-CSF. All ^{125}I-G-CSF preparations were used within 2 weeks.

Measurement of G-CSF-R Down-Modulation

Cells were incubated with G-CSF (5 nM) at 37°C for various periods of time up to 2 h. After G-CSF treatment, cells were washed twice with cold PBS, resuspended in the receptor binding buffer (described above), and used for ^{125}I-G-CSF-binding assay. Decrease in the G-CSF-binding activity on the cell-surface was taken as a measure of down-modulation of the G-CSF-R from cell-surface due to internalization. To study the effect of PKC inhibitors on G-CSF-R down-modulation, cells were treated with these agents for 30 min prior to their incubation with G-CSF.

Measurement of [^{3}H]Thymidine Incorporation

Incorporation of [^{3}H]thymidine into cellular DNA was measured as an indicator of cell-proliferation. NFS-60 cells in log phase were harvested, washed twice with IMDM, resuspended in IMDM containing 0.2% FCS, and adjusted to a cell-density of 10^5 cells/ml. One hundred microliters (10^4 cells) was added per well in a 96-well cell-culture plate (Falcon), and incubated with G-CSF (5 nM) at 37°C for 24 h. Cells were then pulsed with 0.5 µCi of [^{3}H]thymidine for 4 h, and DNA harvested with five washes of 250 µl water each in a cell harvester (Cambridge Technology, Watertown, MA). [^{3}H]thymidine incorporation was measured in a liquid scintillation counter (LS-6000LL; Beckman Instruments).

Flow Cytometry

Freshly isolated peripheral blood neutrophils were treated with TMB-8 (50 µM, 0.5 h) or HA-100 (20 µM, 0.5 h) and prior to addition of G-CSF (5 nM, 2 h). Cells were washed once with PBS alone and once with PBS containing 0.1% BSA and 0.1% NaN_3 (PBS/BSA). Cells (10^6) were incubated with an anti-CD11b-FITC antibody or an isotype matched, FITC-conjugated antibody (Sigma Chemicals, St. Louis, MO) in 100 µl PBS/BSA at room temperature for 30 min. Cells were washed twice with PBS/BSA and used for flow cytometry immediately (FACScan, Becton Dickinson). Mean signal fluores-

cence was corrected for the autofluorescence of unstained cells and cells incubated with an isotype-matched FITC-labeled Ab.

RESULTS

Effect of G-CSF Treatment on PKC Activity in Neutrophils and Myeloid Cell Lines

Treatment of peripheral blood neutrophils and myeloid cell lines with G-CSF for various periods of time resulted in a significant increase in PKC activity (Fig. 1). In neutrophils, a large, sevenfold increase was detected in 15 min of G-CSF treatment (Fig. 1A). Although this increase was largely reversed in 30 min, the G-CSF-induced PKC activity remained significantly greater than the basal level up to 4 h. In the granulocytic precursor cell line, KG-1, increased PKC activity was detected within 15 min; the maximum, 2.2-fold activity was seen at 1 h of G-CSF treatment (Fig. 1C). In other granulocytic (HL-60, NFS-60) and myelomonocytic (U-937, WEHI-3B) cell lines also, a moderate, but significant (0.5–0.6-fold) increase in PKC activity was observed between 15 min and 4 h of G-CSF treatment (Fig. 1B,D,E,F). Substrate-phosphorylation was specific to PKC activation, as it was inhibited by PKC 19-36, a PKC-specific inhibitor. The basal levels of PKC activity in neutrophils and KG-1 cells were the lowest among cell types tested. The highest basal activity of PKC was observed in HL-60 cells. Modest basal levels of PKC activity were observed in NFS-60, U-937, and WEHI-3B cells. All the cells showed significant PKC activation in response to PMA (10 μM, 20 min), and this induction was comparable to that due to G-CSF treatment (Fig. 1).

Effect of PKC Inhibitors on G-CSF-Induced PKC Activity in HL-60 Cells

To confirm that the substrate-phosphorylating activity induced by G-CSF was indeed due to PKC, we studied the effects of various PKC inhibitors on PKC activity in G-CSF-treated HL-60 cells. Chelerythrine chloride and HA-100, the direct, specific inhibitors of PKC, BAPTA, a chelator of intracellular calcium, and TMB-8, an inhibitor of intracellular calcium release, blocked G-CSF-induced PKC activation completely, confirming that G-CSF-induced substrate-phosphorylating activity was that of PKC (Fig. 2). To determine if G-CSF-activated pathways involving tyrosine kinases and PKC were related events, HL-60 cells were treated with methyl-dihydroxycinnamate (MDHC), a potent, cell-permeable tyrosine kinase inhibitor, followed by G-CSF, and PKC activity measured. MDHC treatment of HL-60 cells resulted in a complete inhibition of G-CSF-induced PKC activation, suggesting that PKC activation may occur downstream of tyrosine kinase activation in G-CSF-R signal transduction.

Effect of PKC Inhibitors on [^{125}I]G-CSF Binding to HL-60 Cells

To determine whether changes in HL-60 PKC activity after treatment with various PKC inhibitors were effects of post-receptor mechanisms or ligand-receptor interaction, [^{125}I]G-CSF binding to G-CSF-R was measured in cells treated with these agents. None of the PKC inhibitors, chelerythrine chloride, HA-100, BAPTA or TMB-8 showed any significant effect on G-CSF binding to its receptor, demonstrating that the PKC inhibitors affected PKC activity by post-receptor mechanisms (Table I).

Effect of PKC Inhibitors on Down-Modulation of G-CSF-Receptors on HL-60 Cells

Ligand-induced internalization is a phenomenon common to several cytokine receptors, including the G-CSF-R. To verify the ability of G-CSF to down-modulate its receptors, HL-60 cells were first treated with G-CSF (5 nM) for 0–2 h and used in the [^{125}I]G-CSF-binding assay to measure the number of G-CSF-R remaining on the cell surface. G-CSF down-modulated its receptors almost completely (~95%) within 0.25 h (data not shown). This down-modulation was further maintained for at least 2 h after G-CSF treatment. To evaluate the role of PKC in G-CSF-induced receptor down-modulation, HL-60 cells were treated with different doses of PKC inhibitors for 30 min, followed by G-CSF (5 nM) for 15 min, and [^{125}I]G-CSF-binding assay performed. None of the PKC inhibitors showed any inhibitory effect on G-CSF induced receptor down-modulation (Fig. 3). G-CSF-receptors were down-modulated completely (93–99%) within 15 min of G-CSF treatment in the presence or absence of PKC inhibitors, suggesting that ligand-induced G-CSF-R down-modulation was independent of PKC activation.

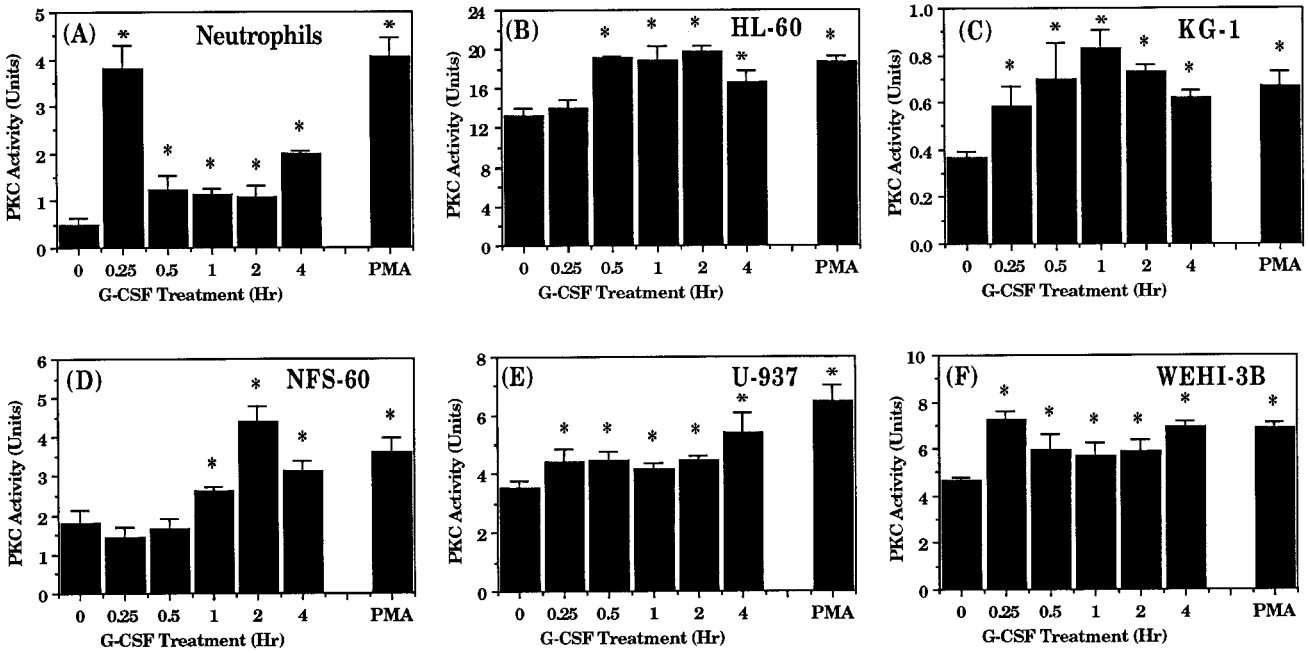

Fig. 1. Effect of G-CSF-treatment on PKC activity in myeloid cells. Neutrophils, HL-60, KG-1, NFS-60, U-937, and WEHI-3B cells were treated with 5 nM rhG-CSF for 0–4 h at 37°C, and lysed. PKC from cell lysates was partially purified over DE52 columns as described in Materials and Methods. Aliquots of PKC were incubated first with 20 μM PKC inhibitor peptide (PKC 19–36) or lipids at room temperature for 20 min, and then with 250 μM Ac-MBP and 5 μCi/ml [γ^{32}P]ATP at 37°C for 5 min. Aliquots (25 μl) of reaction mixtures were spotted on to phosphocellulose paper-discs, filters washed with 1% phosphoric acid, and incorporated radioactivity measured. PKC activity was represented as pmoles of [γ^{32}P]ATP transferred to Ac-MBP per minute by PKC purified from a million cells. PKC activity in cells treated with 10 μM PMA for 20 min was used as a positive standard. Results are represented as mean ± SD from three independent experiments. *$P \leq 0.05$, Student's *t* test.

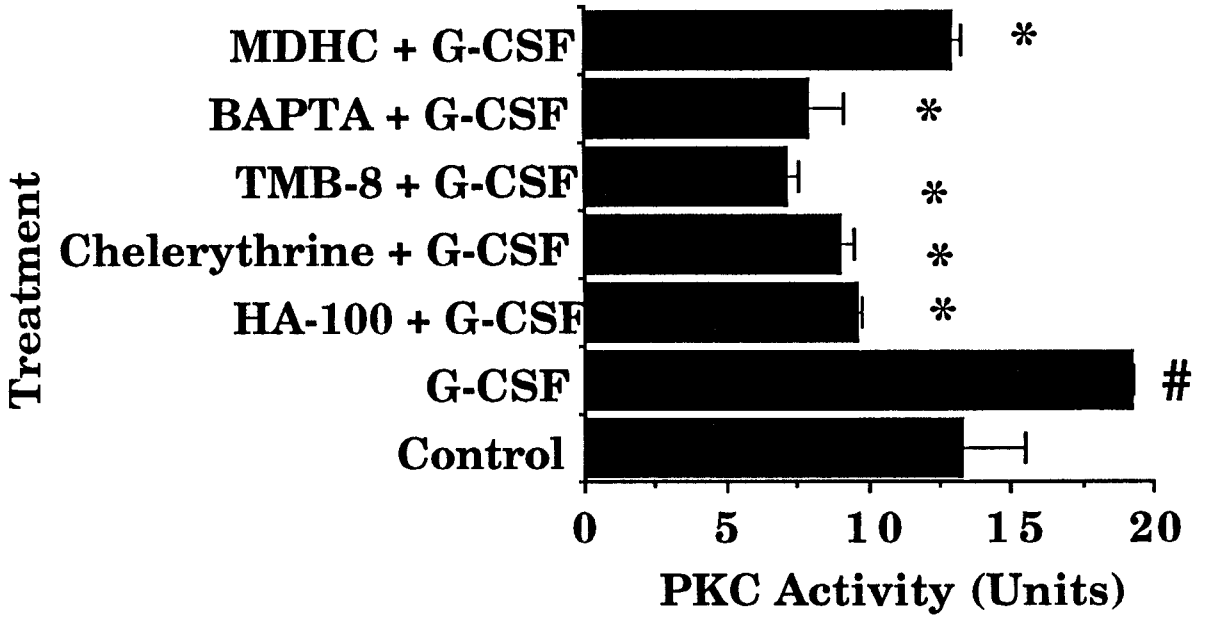

Fig. 2. Effect of PKC and PTK inhibitors on PKC activity in G-CSF-treated HL-60 cells. HL-60 cells were treated with PKC inhibitors—chelerythrine chloride (10 μM), HA-100 (20 μM), BAPTA (10 μM), TMB-8 (50 μM)—or PTK inhibitor—MDHC (100 μg/ml)—at 37°C for 30 min, followed by G-CSF (5 nM) for 30 min, and PKC activity measured as described. Control cells were incubated for the same length of time, but without any added PKC inhibitors or G-CSF. Results are expressed as mean ± SD from three independent experiments. *$P \leq 0.05$, Student's *t* test, compared with cells treated with G-CSF alone. #$P \leq 0.05$, Student's *t* test, compared with untreated control cells.

TABLE I. Effect of PKC Inhibitors on [^{125}I]G-CSF-Binding to HL-60 Cells

Treatment	[^{125}I]G-CSF -binding (cpm/10^6 cells)	% Inhibition
Control	4461 ± 104	0.00 ± 2.34
TMB-8 (50 μM)	4224 ± 57	5.32 ± 1.28
BAPTA (10 μM)	4346 ± 102	2.59 ± 2.29
HA-100 (20 μM)	4611 ± 267	−3.35 ± 5.98
Chelerythrine chloride (10 μM)	4561 ± 685	−2.22 ± 1.98

HL-60 cells were incubated at 37°C for 30 min with or without (control) different PKC inhibitors and ^{125}I-G-CSF binding assay performed as described in Materials and Methods. Data are represented as mean ± SD from three independent experiments.

Effect of PKC Inhibitors on [^{3}H]Thymidine Uptake by NFS-60 Cells

To test the potential role of G-CSF-induced PKC in cell-proliferation, we studied the proliferation of NFS-60 cells in the presence or absence of G-CSF and PKC inhibitors (Fig. 4). Treatment of NFS-60 cells with chelerythrine chloride and TMB-8 resulted in a dose-dependent, anti-proliferative response. However, these treatments did not alter the ratio of G-CSF-dependent to G-CSF-independent thymidine uptake. Similarly, HA-100 and BAPTA did not change this ratio, suggesting that G-CSF-induced proliferation of NFS-60 cells was independent of PKC activation.

Effect of PKC Inhibitors on G-CSF-Induced CD11b Expression on Peripheral Blood Neutrophils

To study the effect of G-CSF on CD11b expression on neutrophils, these cells were treated with G-CSF (5 nM) for various periods of time up to 2 h, and CD11b expression studied by flow cytometry (data not shown). G-CSF up-regulated expression of CD11b on neutrophils by 44% in 2 h (Table II). To investigate a potential involvement of PKC in this induction, neutrophils were treated first with HA-100 (20 μM, 0.5 h) or TMB-8 (50 μM, 0.5 h), followed by G-CSF (5 nM, 2 h), and CD11b expression measured. Treatment with HA-100 and TMB-8 resulted in a 34% and 29% decrease in G-CSF-induced CD11b expression, respectively, indicating a partial correlation between PKC activation and CD11b up-regulation in neutrophils.

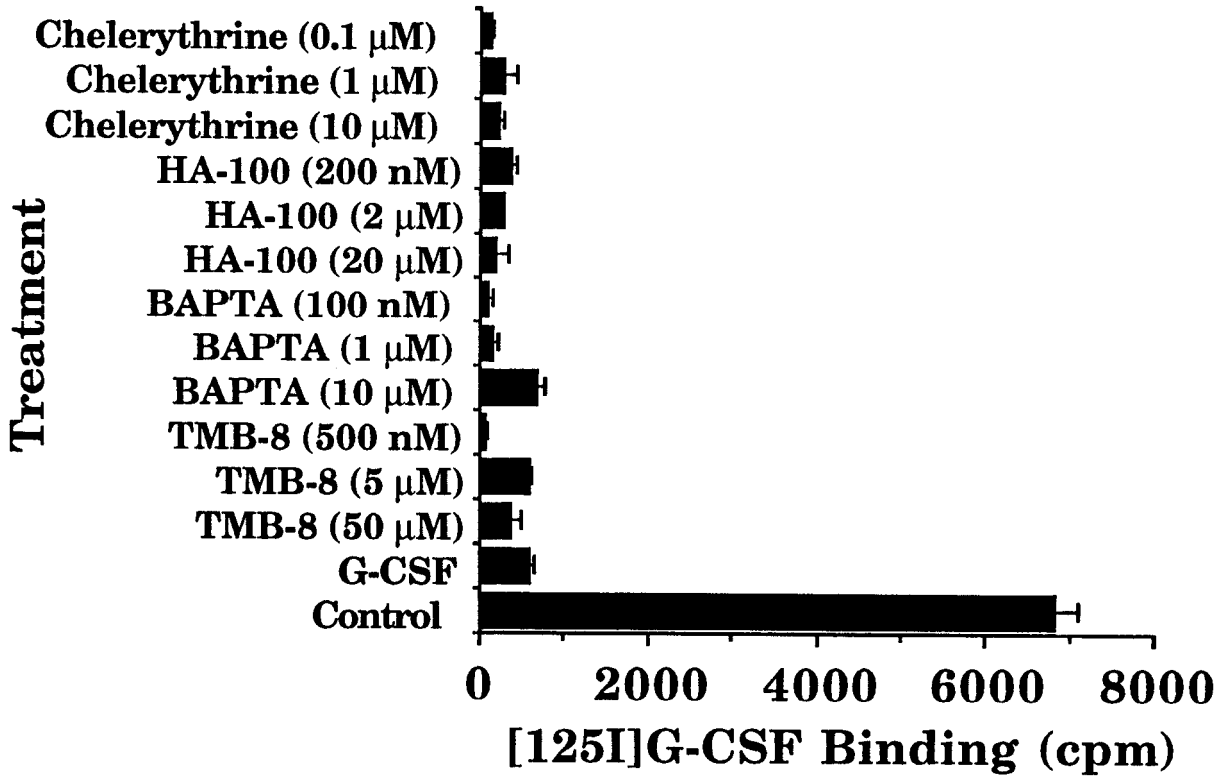

Fig. 3. Effect of PKC-inhibitors on down-modulation of G-CSF-receptors on HL-60 cells. HL-60 cells were treated with different doses of PKC inhibitors at 37°C for 30 min, followed by G-CSF (5 nM) for 15 min. [^{125}I]G-CSF-binding assay was performed as described in Materials and Methods. Decrease in G-CSF-binding activity on the cell-surface was taken as a measure of down-modulation of the G-CSF-R from cell-surface due to internalization. Results are represented as mean ± SD from three independent experiments.

DISCUSSION

G-CSF, a member of the family of myeloid growth factors, supports hematopoiesis and regulates effector functions in neutrophils. Although a single class of high-affinity G-CSF-R has been detected on several cell types, its cDNA cloned and chromosomal location identified, the mechanisms by which G-CSF exerts its cellular effects are largely unknown. Considerable effort in understanding the events subsequent to G-CSF/G-CSF-R interaction has fetched the knowledge of tyrosine kinase activation in myeloid cells. However, other signaling mechanisms, similar to those seen in other cytokine receptor systems, remain unexplored for the G-CSF-R. In this study, we explored PKC modulation as a potential mechanism of G-CSF-R signal transduction.

PKC activation appears to be a major control point in cellular responses, as distinct cytokines utilize this pathway for intracellular signaling. The development of in vitro assays for measuring PKC activity has been explored extensively, using diverse experimental variables including ionic conditions, lipid contents, substrate sources, pharmacological agonists and antagonists, and kinetics of activation [reviewed in ref. Epand, 1994]. The components of the PKC assay used in this study have been used successfully and reproducibly for measuring PKC activity in other systems [House and Kemp, 1987; Yasuda et al., 1990].

In this report, we describe activation of protein kinase-C in G-CSF-treated myeloid cell lines and peripheral blood neutrophils, and provide direct evidence for the involvement of this pathway in G-CSF-R signal transduction. G-CSF-treatment of cells resulted in a 0.5–7-fold increase in PKC activity over basal levels. The specificity of PKC activation was confirmed by its inhibition by PKC 19-36, a peptide whose sequence was derived from the pseudosubstrate region of PKC. That the substrate-phosphorylating activity induced by G-CSF was indeed due to PKC was further confirmed using direct (chelerythrine chloride, HA-100) and in-

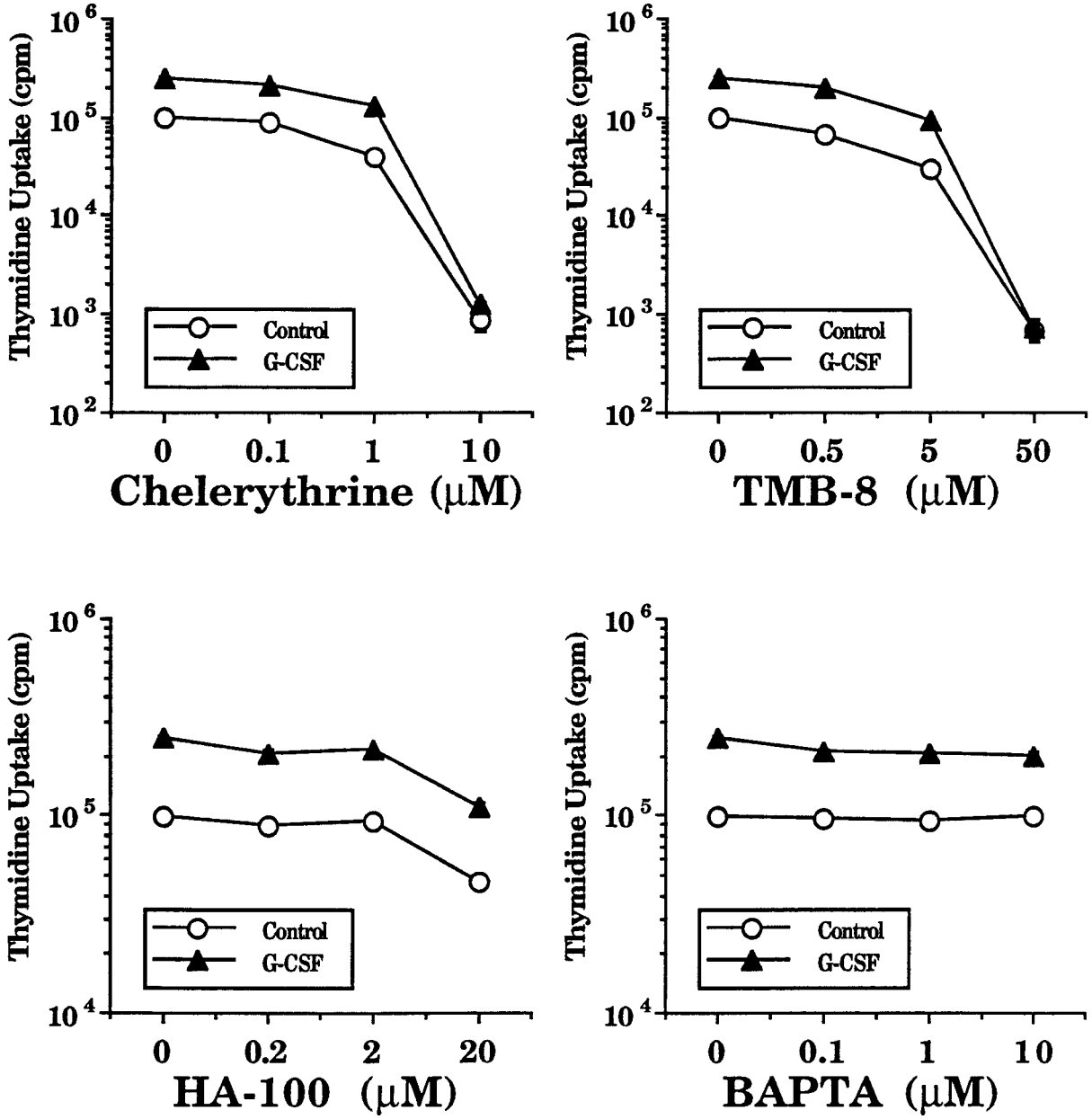

Fig. 4. Effect of PKC inhibitors on [^{3}H]thymidine uptake by NFS-60 cells. NFS-60 cells (10^4) in log phase were suspended in IMDM containing 0.2% FCS, and incubated with rhG-CSF (5 nM) and/or different doses of PKC inhibitors at 37°C for 24 h. Cells were pulsed with 0.5 μCi [^{3}H]thymidine for 4 h, DNA harvested with five washes of 250 μl water, and [^{3}H]thymidine incorporation measured in a liquid scintillation counter as described in Materials and Methods. Results are indicated as mean ± SD from three independent experiments (the small standard deviations are within symbols, and are not clearly evident on the exponential scales used for the graphs).

direct (TMB-8, BAPTA) inhibitors of PKC. Unlike the non-specific inhibitors (H-7, staurosporine, gossypol) that bind the ATP-binding site in the catalytic domain of PKC, chelerythrine is a highly selective and specific inhibitor which blocks the diglyceride binding site within the regulatory domain of PKC [Herbert et al., 1990]. Treatment of HL-60 cells (5 μM, 6 h) with chelerythrine has no adverse effects on cell viability [Jarvis et al., 1994]. It is also used with platelets, endothelial cells, and basophils [Fujimoto and McEver, 1993; Patella et al., 1995]. HA-100 (1-(5-isoquinolinesulfonyl)-piperazine) competes with ATP to bind PKC, but does not affect activities of ATPases, adenylate cyclase or guanylate cyclase even at millimolar

TABLE II. Effect of PKC Inhibitors on G-CSF-Induced CD11b Expression on Peripheral Blood Neutrophils

Pre-treatment	Fluorscence (units)		G-CSF/control ratio
	Control	G-CSF	
None	211.30 ± 10.91	314.56 ± 6.24	1.49 ± 0.05
TMB-8 (50 μM)	252.03 ± 2.88	283.90 ± 1.91	1.12 ± 0.01*
HA-100 (20 μM)	197.64 ± 2.53	184.66 ± 0.80	0.93 ± 0.01*

Freshly isolated peripheral blood neutrophils were incubated with or without G-CSF (5 nM) at 37°C for 2 h, and CD11b surface expression measured by flow cytometry as described in Materials and Methods. Separately, neutrophils were pre-treated with 50 μM TMB-8 or 20 μM HA-100 at 37°C for 30 min, followed by 5 nM G-CSF for 2 h, and CD11b expression measured. Specific fluorescence in arbitrary units was calculated by correcting for cell-autofluorescence and isotype-matched FITC-labeled antibody fluorescence of stained cells. Data are represented as mean ± SEM from two independent experiments. *$P \leq 0.05$, Student's *t* test.

concentrations, and has been used with endothelial cells (10 μM) [Hagiwara et al., 1987; Kuzuya and Kinsella, 1994]. TMB-8, an inhibitor of intracellular calcium release, inhibits neutrophil chemotaxis and PMA-induced leukocyte histamine release (at 2–200 μM) [Srivastava et al., 1994; Bergstrand et al., 1992]. It has been used with myeloid cell lines M1-A5 and U-937 [MacIntyre and Pope, 1991; Li et al., 1993]. BAPTA, a chelator of intracellular calcium, has been used with human neutrophils (10–100 μM), HL-60, and U-937 cells [Suchard and Boxer, 1994; Elsner et al., 1996; Yoshida et al., 1993; Natarajan and Iwamoto, 1994].

Like the PKC inhibitors used in the current study, methyl 2,5-dihydroxycinnamate (MDHC), a potent, cell-permeable, specific inhibitor of protein tyrosine kinases [Umezawa et al., 1990], also blocked PKC activation in G-CSF-treated HL-60 cells, suggesting that PKC activation may occur downstream of PTK activation in these cells. The correlation between PTK activation and PKC induction has been observed in several cell types. In B lymphocytes, CD40-mediated PKC activation and translocation is dependent on PTK activation [Ren et al., 1994]. In thrombin-activated platelets, tyrosine phosphorylation leads to phosphatidylinositol 3,4-bisphosphate synthesis and PKC activation [Yatomi et al., 1994]. In vascular endothelial growth-factor-treated endothelial cells, stimulation of PTK activity results in phospholipase-C phosphorylation, PKC activation, and cell proliferation [Xia et al., 1996]. Although the exact mechanism(s) of PTK/PKC activation cascade(s) remain to be fully understood, they seem to involve some modifications of different phospholipases [for review, see Halenda et al., 1996; Natarajan et al., 1996]. It is possible that in agonist-stimulated cells, the activated cytoplasmic PTKs are recruited to the membrane receptors, and through protein phosphorylation, they may increase the efficiency of coupling of the receptors with phospholipases [Garland, 1992].

Ligand-induced down-modulation is common to several growth factor receptor systems, including the G-CSF-R. 'Trans-down-modulation' due to interactions of other ligands and receptors is also common among cytokine receptors. TNF down-regulates G-CSF-R expression on human myeloid leukemic blasts and neutrophils by 70%. While mechanisms underlying G-CSF-induced down-modulation of G-CSF-R are not clear, those resulting in TNF-induced trans-down-modulation of G-CSF-R appear to involve the PKC pathway. The TNF-effect can be mimicked by PMA but not GM-CSF, IL-2 or IL-6 [Elbaz et al., 1991]. To determine if G-CSF-induced down-modulation of G-CSF-R also involves the PKC pathway, we studied the effects of several PKC inhibitors on G-CSF-R down-modulation in HL-60 cells. None of the inhibitors showed any effect on G-CSF-R down-modulation, indicating that ligand-induced down-modulation of the G-CSF-R is independent of PKC pathway.

G-CSF promotes proliferation of NFS-60 cells and myeloid precursors. To determine if G-CSF-mediated PKC activation may have a role in proliferation of immature myeloid cells, we studied the effects of PKC inhibitors on thymidine uptake by NFS-60 cells. None of the PKC inhibitors showed any effect on the G-CSF-specific proliferation of NFS-60 cells, suggesting that G-CSF-induced proliferation of granulocytic precursors may involve mechanisms other than PKC activation.

In neutrophils, which are terminally differentiated cells, G-CSF enhances cell survival and effector functions. Neutrophils express CD11b,

which, together with CD18, forms the complement receptor-3. It plays a fundamental role in binding un-opsonized bacteria, yeasts, fibrinogen and factor X, and promotes neutrophil cell-cell adhesion as well as neutrophil-endothelial cell adhesion [Wright and Jong, 1986; Ross et al., 1985, 1987; Wright et al., 1988; Buyon et al., 1988; Arnaout et al., 1988]. In this study, treatment of neutrophils with HA-100 and TMB-8 resulted in a moderate decrease in G-CSF-induced CD11b expression, suggesting a partial correlation between PKC activation and CD11b induction. Similar correlation is also seen in neutrophils migrating across IL-1β-pretreated endothelial cells (IL-1-EC); these neutrophils show a rapid increase in $[Ca^{2+}]i$, associated with up-regulation of CD11b on the surface, and treatment with BAPTA (25 μM, 30 min) abrogates this up-regulation of CD11b [Kuijpers et al., 1992].

The physiological significance of PKC activation specific to the G-CSF/G-CSF-R interaction remains to be understood. In general, PKC is considered to play a role in myeloid cell survival and proliferation. Chelerythrine treatment (5 μM, 6 h) of HL-60 cells abolishes their clonogenicity, whereas treatment of neutrophils with PKC inhibitors results in loss of cell survival in culture [Jarvis et al., 1994; Adachi et al., 1993]. Differential expression, activation, and subcellular distribution of PKC isoforms are also implicated in increased CD34 expression, lineage commitment, and maturation of myelomonocytic precursors [Fackler et al., 1992; Shearman et al., 1993; Rossi et al., 1996].

In conclusion, our results demonstrate the involvement of PKC in G-CSF-R signal transduction, and suggest a common signaling pathway in myeloid cells of granulocytic and monocytic lineages.

REFERENCES

Adachi S, Kubota M, Matsubara K, Wakazono Y, Hirota H, Kuwakado K, Akiyama Y, Mikawa H (1993): Role of protein kinase C in neutrophil survival enhanced by granulocyte colony-stimulating factor. Exp Hematol 21: 1709–1713.

Arnaout M, Lanier L, Faller D (1988): Relative contribution of the leukocyte molecules Mo1, LFA-1, and p150,95 (Leu M5) in adhesion of granulocytes and monocytes to vascular endothelium is tissue- and stimulus-specific. J Cell Physiol 137:305–312.

Avalos BR (1996): Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood 88:761–777.

Begley CG, Metcalf D, Nicola NA (1988): Binding characteristics and proliferative action of purified granulocyte colony-stimulating factor (G-CSF) on normal and leukemic human promyelocytes. Exp Hematol 16:71–79.

Bergstrand H, Lundquist B, Karabelas K, Michelsen P (1992): Modulation of human basophil histamine release by protein kinase C inhibitors differs with secretagogue and with inhibitor. J Pharmacol Exp Ther 260:1028–1037.

Berridge MJ (1993): Inositol triphosphate and calcium signalling. Nature 361:315–325.

Buyon J, Abramson S, Philips M, Slade S, Ross G, Weissmann G, Winchester R (1988): Dissociation between increased surface expression of Gp165/95 and homotypic neutrophil aggregation. J Immunol 140:3156–3160.

Clark SC, Kamen R (1987): The human hematopoietic colony-stimulating factors. Science 236:1229–1237.

Corey SJ, Burkhardt AL, Bolen JB, Geahlen RL, Tkatch LS, Tweardy DJ (1994): Granulocyte colony-stimulating factor receptor signaling involves the formation of a three-component complex with lyn and syk protein-tyrosine kinases. Proc Natl Acad Sci USA 91:4683–4687.

Deshpande RV, Peterson RHF, Moore MAS (1997): Protein tyrosine kinases in granulocyte colony stimulating factor receptor signal transduction, myeloid cell proliferation, and neutrophil activation. Life Sci 60:587–604.

Elbaz O, Budel LM, Hoogerbrugge H, Touw IP, Delwel R, Mahmoud LA, Lowenberg B (1991): Tumor necrosis factor downregulates granulocyte-colony-stimulating factor receptor expression on human acute myeloid leukemia cells and granulocytes. J Clin Invest 87:838–841.

Elsner J, Dichmann S, Dobos GJ, Kapp A (1996): Actin polymerization in human eosinophils, unlike human neutrophils, depends on intracellular calcium mobilization. J Cell Physiol 167:548–555.

Epand RM (1994): In vitro assays of protein kinase C activity. Anal Biochem 218:241–247.

Fackler MJ, Civin CI, May WS (1992): Up-regulation of surface CD34 is associated with protein kinase C-mediated hyperphosphorylation of CD34. J Biol Chem 267:17540–17546.

Fujimoto T, McEver RP (1993): The cytoplasmic domain of P-selectin is phosphorylated on serine and threonine residues. Blood 82:1758–1766.

Garland LG (1992): New pathways of phagocyte activation: The coupling of receptor-linked phospholipase D and the role of tyrosine kinase in primed neutrophils. FEMS Microbiol Immunol 5:229–237.

Hagiwara M, Inagaki M, Watanabe M, Ito M, Onoda K, Tanaka T, Hidaka H (1987): Selective modulation of calcium-dependent myosin phosphorylation by novel protein kinase inhibitors, isoquinolinesulfonamide derivatives. Mol Pharmacol 32:7–12.

Halenda SP, Wu H, Jones AW, Shukla SD (1996): Phospholipase D in platelets and megakaryocytic cells. Chem Phys Lipids 80:21–26.

Herbert JM, Augereau JM, Gleye J, Maffrand JP (1990): Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 172:993–999.

House C, Kemp BE (1987): Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. Science 238:1726–1728.

Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S (1994): Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. Cancer Res 54:1707–1714.

Kuijpers TW, Hoogerwerf M, Roos D (1992): Neutrophil migration across monolayers of resting or cytokine-activated endothelial cells. J Immunol 148:72–77.

Kuzuya M, Kinsella JL (1994): Reorganization of endothelial cord-like structures on basement membrane complex (Matrigel): Involvement of transforming growth factor beta 1. J Cell Physiol 161:267–276.

Li Q, Tallant A, Cathcart MK (1993): Dual Ca2+ requirement for optimal lipid peroxidation of low density lipoprotein by activated human monocytes. J Clin Invest 91: 1499–1506.

MacIntyre JP, Pope BL (1991): The involvement of protein kinase C, calcium, and 5-lipoxygenase in the production of tumor necrosis factor by a cloned interleukin-3 dependent cell line with natural cytotoxic activity. Int J Immunopharmacol 13:175–184.

Matsuda T, Hirano T (1994): Association of p72 tyrosine kinase with Stat factors and its activation by interleukin-3, interleukin-6, and granulocyte colony-stimulating factor. Blood 83:3457–3461.

Mischak H, Kolch W, Goodnight J, Davidson WF, Rapp U, Rose-John S, Mushinski JF (1991): Expression of protein kinase C genes in hemopoietic cells is cell-type- and B cell-differentiation stage specific. J Immunol 147:3981–3987.

Natarajan V, Iwamoto GK (1994): Lipopolysaccharide-mediated signal transduction through phospholipase D activation in monocytic cell lines. Biochim Biophys Acta 1213:14–20.

Natarajan V, Scribner WM, Vepa S (1996): Regulation of phospholipase D by tyrosine kinases. Chem Phys Lipids 80:103–116.

Nicholson SE, Oates AC, Harpur AG, Ziemiecki A, Wilks AF, Layton JE (1994): Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation. Proc Natl Acad Sci USA 91:2985–2988.

Nicola NA (1987): Granulocyte colony-stimulating factor and differentiation-induction in myeloid leukemic cells. Int J Cell Cloning 5:1–15.

Nicola NA, Metcalf D (1984): Binding of the differentiation-inducer, granulocyte-colony-stimulating factor, to responsive but not unresponsive leukemic cell lines. Proc Natl Acad Sci USA 81:3765–3769.

Nishizuka Y (1986): Studies and perspectives of protein kinase C. Science 233:305.

Nishizuka Y (1988): The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665.

Ogita K, Miyamoto S, Yamaguchi K, Koide H, Fujisawa N, Kikkawa U, Sahara S, Fukami Y, Nishizuka Y (1992): Isolation and characterization of delta-subspecies of protein kinase C from rat brain. Proc Natl Acad Sci USA 89:1592–1596.

Patella V, Casolaro V, Ciccarelli A, Pettit G, Columbo M, Marone G (1995): The antineoplastic bryostatins affect human basophils and mast cells differently. Blood 85: 1272–1281.

Ren CL, Fu SM, Geha RS (1994): Protein tyrosine kinase activation and protein kinase C translocation are functional components of CD40 signal transduction in resting human B cells. Immunol Invest 23:437–448.

Ross GD, Cain JA, Lachmann PJ (1985): Membrane complement receptor type three (CR_3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. J Immunol 134:3307–3315.

Ross G, Cain J, Myones B, Newman S, Lachmann P (1987): Specificity of membrane complement receptor type three (CR3) for beta-glucans. Complement 4:61–74.

Rossi F, McNagny KM, Smith G, Frampton J, Graf T (1996): Lineage commitment of transformed haematopoietic progenitors is determined by the level of PKC activity. EMBO J 15:1894–1901.

Shearman MS, Heyworth CM, Dexter TM, Haefner B, Owen PJ, Whetton AD (1993): Haemopoietic stem cell development to neutrophils is associated with subcellular redistribution and differential expression of protein kinase C subspecies. J Cell Sci 104:173–180.

Shieh JH, Peterson RHF, Moore MAS (1991): Modulation of granulocyte colony-stimulating factor receptors on murine peritoneal exudate macrophages by tumor necrosis factor-α. J Immunol 146:2648–2653.

Srivastava S, Khanna N, Mathur A, Chaturvedi UC (1994): Role of calcium in neutrophil activation by Japanese encephalitis virus-induced macrophage derived factor. Acta Virol 38:111–116.

Suchard SJ, Boxer LA (1994): Exocytosis of a subpopulation of specific granules coincides with H_2O_2 production in adherent human neutrophils. J Immunol 152:290–300.

Tian SS, Lamb P, Seidel HM, Stein RB, Rosen J (1994): Rapid activation of the STAT3 transcription factor by granulocyte colony-stimulating factor. Blood 84:1760–1764.

Umezawa K, Hori T, Tajima H, Imoto M, Isshiki K, Takeuchi T (1990): Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. FEBS Lett 260:198–200.

Wright SD, Jong MTC (1986): Adhesion-promoting receptors on human macrophages recognize *Escherichia coli* by binding to lipopolysaccharide. J Exp Med 164:1876–1888.

Wright S, Weitz J, Huang A, Levin S, Silverstein S, Loike J (1988): Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. Proc Natl Acad Sci USA 85:7734–7738.

Xia P, Aiello LP, Ishii H, Jiang ZY, Park DJ, Robinson GS, Takagi H, Newsome WP, Jirousek MR, King GL (1996): Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. J Clin Invest 98:2018–2026.

Yasuda I, Kishimoto A, Tanaka S, Tominaga M, Sakurai A, Nishizuka Y (1990): A synthetic peptide substrate for selective assay of protein kinase C. Biochem Biophys Res Commun 166:1220–1227.

Yatomi Y, Ozaki Y, Satoh K, Kume S (1994): Synthesis of phosphatidylinositol 3,4-bisphosphate is regulated by protein-tyrosine phosphorylation but the p85 alpha subunit of phosphatidylinositol 3-kinase may not be a target for tyrosine kinases in thrombin-stimulated human platelets. Biochim Biophys Acta 1212:337–344.

Yoshida A, Ueda T, Takauji R, Liu YP, Fukushima T, Inuzuka M, Nakamura T (1993): Role of calcium ion in induction of apoptosis by etoposide in human leukemia HL-60 cells. Biochem Biophys Res Commun 196:927–934.