Enhancement of chemotactic peptide-induced activation of phosphoinositide 3-kinase by granulocyte–macrophage colony-stimulating factor and its relation to the cytokine-mediated priming of neutrophil superoxide-anion production

Takeshi KODAMA*†, Kaoru HAZEKI*, Osamu HAZEKI‡, Taro OKADA‡ and Michio UI*§¹

*The Ui Laboratory, the Institute of Physical and Chemical Research, Hirosawa 2-1, Wako-shi 351-0198, Japan, †Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Kagasuno 463-10, Kawauchi-cho, Tokushima 771-0130, Japan, ‡Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan, and §The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, Japan

Incubation of human neutrophils with a chemotactic peptide [*N*-formylmethionyl-leucylphenylalanine (fMLP)] gave rise to an increase in the phosphoinositide 3-kinase (PI3K) activity, phosphorylation of p47^{phox} and superoxide-anion (O₂^{•−}) generation in the same fMLP-concentration-dependent manner. These responses to fMLP were markedly enhanced when the cells had been incubated for 10 min before the addition of fMLP with increasing concentrations of granulocyte–macrophage colony-stimulating factor (GM-CSF) that were only slightly effective themselves. Wortmannin, an inhibitor of PI3K, suppressed all of these fMLP actions in the same concentration-dependent manner in either GM-CSF-primed or non-primed cells. Sustained activation of protein kinase C by the addition of PMA caused marked phosphorylation of p47^{phox} and respiratory burst itself without activation of PI3K. This strong action of PMA was not primed by GM-CSF. The chemotactic peptide was without effect in pertussis-toxin-treated cells, indicating that its actions are mediated by $\beta\gamma$ -subunits liberated from toxin-susceptible heterotrimeric G_i proteins (G $\beta\gamma$). Thus one of the mechanisms of GM-

CSF-mediated priming of fMLP-induced respiratory burst is synergistic activation of wortmannin-sensitive PI3K by G $\beta\gamma$ in the presence of tyrosine-phosphorylated proteins in GM-CSF-treated cells, as recently indicated in a cell-free system [Kurosu, Maehama, Okada, Yamamoto, Hoshino, Fukui, Ui, Hazeki and Katada (1997) *J. Biol. Chem.* 272, 24252–24256]. GM-CSF primed fMLP-induced MAP (mitogen-activated protein) kinase activation enormously as well. The MAP kinase activation was primed even in the presence of wortmannin, indicating that PI3K was not the sole site where tyrosine kinase-related and G $\beta\gamma$ -mediated intracellular signals converge to elicit the priming. The GM-CSF priming of fMLP-induced PI3K activation and O₂^{•−} generation was much smaller in magnitude in neutrophils in which cAMP accumulated upon incubation with prostaglandin E₁ than in the cells without the nucleotide accumulation. Thus the GM-CSF priming site, in addition to PI3K, might be just the target of cAMP-dependent protein kinase A in fMLP-initiated signalling cascades or could be localized immediately downstream thereof.

INTRODUCTION

The respiratory burst is one of the marked responses of neutrophils to stimulation of their chemokine receptors by a chemotactic peptide, *N*-formylmethionyl-leucylphenylalanine (fMLP). The intracellular signals arising from the fMLP receptors have been proposed to be mediated by heterotrimeric G-protein G $\alpha\beta\gamma$ and phosphoinositide 3-kinase (PI3K), eventually leading to NADPH oxidase activation. It is because the fMLP-induced respiratory burst is accompanied by PI3K activation; both responses are abolished by prior exposure of the cells to pertussis toxin, a blocking agent of receptor–G_i interaction, or to wortmannin, a selective inhibitor of the kinase (see [1] for a review). In fact, G $\beta\gamma$ originating from pertussis-toxin-susceptible G_i is capable of activating certain families of PI3K [2–6].

The first and best characterized subfamily of PI3K comprises a heterodimer containing an 85 kDa regulatory subunit (p85 α or

p85 β) and a 110 kDa catalytic subunit (p110 α or p110 β). The p85 subunit possesses SH2 (Src homology 2) domains to which tyrosine-phosphorylated peptides with the consensus sequence of YXXM bind, thereby increasing the catalytic activity of the associated p110 subunit (see [7–9] for reviews). Thus the p110/p85 dimeric enzyme must be activated via tyrosine-kinase-related receptors that are responsible for phosphorylation of cellular proteins on tyrosine residues.

Our previous results have suggested that the same dimeric PI3K is activated by G $\beta\gamma$ as well. When lysates prepared from human monocytic THP-1 cells were applied to a MonoQ column, p85-containing fractions of the eluate displayed a PI3K activity that increased upon the addition of G $\beta\gamma$, especially in the presence of a synthetic tyrosine-phosphorylated peptide with the abovementioned p85-binding motif [10]. The p110 β –p85 dimer, but not p110 α –p85, purified from rat liver, was also activated synergistically by combined addition of G $\beta\gamma$ with the tyrosine-

Abbreviations used: ERK, extracellular-signal-regulated kinase; fMLP, *N*-formylmethionyl-leucylphenylalanine; G_i or G $\alpha\beta\gamma$, $\alpha\beta\gamma$ -heterotrimeric G-protein susceptible to pertussis toxin; G $\beta\gamma$, $\beta\gamma$ -subunits of G_i; GM-CSF, granulocyte–macrophage colony-stimulating factor; GF109203X, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide; KR-Hepes, Krebs–Ringer/Hepes medium; IBMX, 3-isobutyl-1-methylxanthine; LY294002, 2-(4-morpholinyl)-8-phenylchromone; MAP kinase, mitogen-activated protein kinase; PD98059, 2-(2'-amino-3'-methoxyphenyl)oxanaphthalen-4-one; PI3K, phosphoinositide 3-kinase.

¹ To whom correspondence should be addressed (e-mail ui@rinshoken.or.jp).

phosphorylated peptide [11]. A single subfamily of PI3K is very likely, therefore, to function at the crosstalk point at which G-protein-coupled receptor- and tyrosine kinase-related-receptor-initiated signalling pathways converge.

The purpose of the present study was to address this issue by examining the involvement of such PI3K activation in similarly synergistic induction of the respiratory burst in response of granulocyte-macrophage colony-stimulating factor (GM-CSF)-primed human neutrophils to fMLP. It has been well documented that neutrophils become 'primed' by prior exposure to cytokines such as GM-CSF or tumour necrosis-factor- α , whose receptors are coupled to tyrosine kinases (see [12–14] for reviews). The data showed that fMLP-induced activation of PI3K was enhanced in GM-CSF-primed cells in the same wortmannin-susceptible manner as was the chemokine-induced respiratory burst. Much more marked synergism was observed, however, in fMLP-induced mitogen-activated protein (MAP) kinase activation, which was only partly inhibited by wortmannin and virtually not involved at all in the respiratory burst in GM-CSF-primed cells. It would be likely, therefore, that the synergistic activation of PI3K observed is not the sole mechanism by which fMLP-induced respiratory burst is primed by GM-CSF.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: dextran T500 and Ficoll–Paque from Pharmacia Biotech; fMLP, PMA, cytochrome *c*, di-isopropyl fluorophosphate and BSA from Sigma; GF109203X and PD98059 from Calbiochem; genistein from Nakalai tesque (Kyoto, Japan); recombinant human GM-CSF from Genzyme; [32 P]P_i and [γ - 32 P]ATP from DuPont–NEN; monoclonal anti-phosphotyrosine antibody (PY-20) from Santa Cruz; rabbit polyclonal anti-phosphotyrosine antibody from Chemicon (Temecula, CA, U.S.A.); monoclonal antibody against the 85 kDa regulatory subunit of PI3K (α p85) from Upstate Biotechnology (Lake Placid, NY, U.S.A.). MAP kinase (ERK) and protein kinase C enzyme assay kits were both obtained from Amersham Life Science. Wortmannin and pertussis toxin were kindly given by Dr. Y. Matsuda (Kyowa Hakko Kogyo Co.) and M. Tamura (Kaken Seiyaku Co.) respectively. The stock solution (10 mM) of wortmannin was prepared in DMSO, stored at -20°C and diluted with appropriate buffer immediately before use. Rabbit anti-p47^{phox} antibody was prepared as described in [15].

Cell preparation and incubation

Venous blood was collected from healthy volunteers (with their informed consent) on heparin (5 units/ml) anticoagulant solution. Neutrophils were isolated by dextran (0.2% sedimentation and Ficoll–Paque gradient centrifugation followed by hypo-osmotic lysis of contaminating erythrocytes. Where indicated, neutrophils were incubated with 100 ng/ml pertussis toxin in RPMI-1640 medium at 37°C for 2 h in a CO₂ incubator. These toxin-treated cells were washed and resuspended in appropriate medium to be subjected to ^{32}P -labelling or further analyses as described below.

The general protocol of the incubation (at 37°C) for analyses of cellular functions was as follows. The incubation medium was Krebs–Ringer/Hepes (KR-Hepes) medium (134 mM NaCl/4.7 mM KCl/1.2 mM KH₂PO₄/1.2 mM MgSO₄/2.5 mM CaCl₂/5 mM glucose/20 mM Hepes, pH 7.4), and the incubation time after the addition of fMLP, PMA or their vehicle was 5 min for measurement of superoxide anion (O₂^{•−}) generation and 30 s for

analyses of PtdInsP₃ production, p47^{phox} phosphorylation and MAP kinase activation. GM-CSF was added, unless otherwise specified as in Figure 9, 10 min before fMLP or PMA or their vehicles. Inhibitors, such as wortmannin, LY294002, GF109203X, PD98059 and genistein, were added 10 min, and prostaglandin E₁ plus IBMX was 3 min, before GM-CSF or its vehicle, where indicated.

O₂^{•−} generation

Neutrophils suspended in KR-Hepes medium containing 80 μM cytochrome *c* (5×10^5 cells in 0.25 ml) were incubated, according to the general protocol described above, for assay of O₂^{•−} generation by measurement of the reduction of the cytochrome based on the difference spectrum at 550–540 nm at the end of incubation. Gradual reduction of cytochrome *c* was observed spontaneously (i.e., without any addition). This spontaneous reduction (O₂^{•−} generation) was subtracted from all the data obtained with additions to provide the net effects of the additions, although there was no significant difference in the spontaneous O₂^{•−} generation between the cells treated with inhibitors or pertussis toxin and the cells without these treatments. The reaction was stopped by adding 0.5 ml of ice-cold Ca²⁺-free KR-Hepes containing 10 mM EDTA, and the mixture was quickly centrifuged (1500 *g* for 10 min) to enable the supernatant to be assayed for cytochrome *c* reduction. The reduction was used for calculation of nmol of O₂^{•−} produced/5 min per 10⁶ cells.

In a typical experiment, the cytochrome *c* reduction was measured in the medium added with 18 units/0.25 ml of superoxide dismutase (Sigma; S2515; from bovine erythrocytes) to find that essentially no cytochrome *c* was reduced under these conditions in either the presence or absence of fMLP (0.1 μM), GM-CSF (10 ng/ml) or PMA (0.1 μM), or to confirm that the reduction without the enzyme addition really reflected O₂^{•−} generation. When the neutrophil O₂^{•−} generation was monitored, with or without these stimulants, from 30 s to 5 min, it increased linearly up to 3 min under all conditions. Though the rate of the generation slightly decreased beyond 3 min, the 5 min values were well correlated with the 3 min values. Thus, either the 5 min data for O₂^{•−} generation are presented in Figures and the Table as a reflection of the rate of the generation, or the data at 30 s are shown, at which time most of the other parameters were measured.

Production of ^{32}P -labelled PtdInsP₃

Production of ^{32}P -labelled PtdInsP₃ was estimated by the technique routinely used in our laboratory [16–19] with only slight modifications. Briefly, neutrophil suspensions (1×10^8 cells in 1 ml of Hepes-buffered medium) labelled with [^{32}P]P_i (150 $\mu\text{Ci}/\text{ml}$) for 30 min at 30°C were, after twice washing, incubated in KR-Hepes (5×10^6 cells in 0.35 ml) with additions of inhibitors (GM-CSF and/or fMLP according to the protocol described above). At the end of incubation, cells were lysed by combining the suspension with 1.55 ml of chloroform/methanol/8% HClO₄ (10:10:1, by vol.), followed by vigorous stirring and further addition with 1 ml of chloroform/8% HClO₄ (1:1, by vol.) to separate the organic phase, which was washed with chloroform-saturated 1% HClO₄ and dried *in vacuo*. The thus-extracted lipids were dissolved in 20 μl of chloroform/methanol (19:1, v/v) and spotted on to a potassium oxalate-impregnated TLC plate (silica-gel 60; Merck). The plate was developed in chloroform/acetone/methanol/acetic acid/water (7:2:5:2:2, by vol.), dried, and radioactive areas revealed with a Fuji BAS2000 bioimaging analyser.

Phosphorylation of p47^{phox}

The ³²P-labelled neutrophils (1×10^8 cells/ml) were kept on ice for 30 min in the Ca²⁺-free Hepes-buffered medium fortified with 3 mM di-isopropyl fluorophosphate. This procedure did not affect O₂⁻ generation nor PI3K activity. The cells were then centrifuged, resuspended in KR-Hepes medium (7×10^6 cells in 0.5 ml) and incubated according to the general protocol described above. The incubation was terminated by the addition of 0.5 ml of ice-cold lysis buffer (the mixture containing 50 mM Tris/HCl, pH 7.4, 2 mM Na₃VO₄, 60 mM NaF, 100 units/ml aprotinin, 2 mM PMSF, 20 μ M leupeptin, 1% sodium deoxycholate, 2% Nonidet P40, 2 mM dithiothreitol, 1% BSA and 10 mM EDTA). The cell lysates were subjected to immunoprecipitation with anti-p47^{phox} antibody and SDS/PAGE on 10% slab gels by the techniques described in detail below. The ³²P content of the p47^{phox} band was quantified by a Fuji BAS2000 bioimaging analyser.

Assay of MAP kinase activity

Neutrophils (3×10^6 cells in 0.2 ml) were incubated according to the general protocol described above. The incubation was terminated by adding 0.2 ml of the modified ice-cold lysis buffer that was prepared by omitting sodium deoxycholate, Nonidet P40 and BSA from the above-mentioned lysis buffer. The supernatant of the lysate, obtained by sonication and centrifugation (15000 *g* for 15 min), was assayed for MAP kinase activity [p42/p44 MAP kinase (ERK) assay kit] by measuring the incorporation of [γ -³²P]ATP into a specific ERK substrate peptide. The reaction was carried out in 25 mM Hepes buffer, pH 7.4, containing 1.5 mM MgCl₂, 2 mM substrate peptide, 0.1 mM sodium orthovanadate, 0.02% sodium azide and 50 μ M [γ -³²P]ATP (1 μ Ci) in a volume of 30 μ l at 37 °C for 30 min. The supernatant was applied to P-81 ion-exchange-chromatography paper, which was washed thoroughly before being dried, and counted for radioactivity in a liquid-scintillation spectrophotometer.

Immunoprecipitation and immunoblotting

Neutrophils (10^8 cells in 1 ml) were kept on ice for 30 min in the Ca²⁺-free Hepes-buffered medium fortified with 3 mM di-isopropyl fluorophosphate. The cells were centrifuged, resuspended in KR-Hepes (7×10^6 cells in 0.5 ml) and incubated according to the protocol described above. The incubation was terminated by the addition of 0.5 ml of ice-cold lysis buffer. The cell lysates obtained by centrifugation (100000 *g*, 15 min) were pre-cleared by the addition of preimmune IgG and Protein G-Sepharose to be kept with gentle rotation for 1 h at 4 °C and subjected to immunoprecipitation by incubation with PY-20 or other antibodies for more than 1 h, which was followed by a second incubation with the further addition with Protein G-Sepharose for a similar length of time. After repeated washings (with the washing solution prepared by omitting protease inhibitors from the above-mentioned lysis buffer), the immunoprecipitates were heated at 100 °C for 3 min in 30 μ l of the sample buffer consisting of 62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% Bromophenol Blue. The proteins thus solubilized were separated by SDS/PAGE on 10% slab gels and transferred electrophoretically to a PVDF membrane (Millipore) at 2 mA/cm² for 40 min. Membranes were blocked, probed with appropriate antibodies and proteins revealed using ¹²⁵I-Protein G. The radioactivity was quantified with a Fuji BAS2000 bioimaging analyser.

Determination of cellular cAMP concentration

The incubation of neutrophils (10^6 cells in 0.15 ml) with various concentrations of prostaglandin E₁ and 50 μ M 3-isobutyl-1-methylxanthine (IBMX), together with other additions according to the above mentioned protocol, was terminated by the addition of HCl at a final concentration of 0.1 M, which was followed by boiling for 3 min and centrifugation. The resultant supernatant was submitted to radioimmunoassay for cAMP as described previously [20].

RESULTS

GM-CSF-mediated priming of fMLP-induced PI3K activation, p47^{phox} phosphorylation and O₂⁻ generation

The chemotactic-peptide-induced respiratory burst in neutrophils was enhanced by GM-CSF, with the strongest effect being observed when the cytokine was added 10 min before fMLP (results not shown). The priming effect of 10 min-pre-added GM-CSF is shown in Figure 1, where the responses of human neutrophils to the maximally effective concentrations (0.1–1 μ M) of fMLP were enhanced by 10 ng/ml of GM-CSF 2–4-fold without significant changes in the least (or the half-maximally) effective concentration of fMLP (Figure 1a). The response to 0.1 μ M fMLP was enhanced progressively when GM-CSF, which was only slightly effective by itself (Figure 1b), had been added at concentrations from 0.1 to 100 ng/ml. In contrast, PMA-induced O₂⁻ generation, although it was more effective than fMLP alone, was never enhanced by the prior addition of GM-CSF (Figure 1c).

Lysates were prepared from PMA-treated and non-treated cells by quick sonication, which was followed by 60 min centrifugation (167000 *g* at 4 °C) to separate membranes from cytosol. These fractions were assayed for protein kinase C by a 15 min incubation with the enzyme assay kit containing 225 μ M synthetic peptide and 1.2 mM [γ -³²P]ATP at 37 °C. The radioactivity incorporated into the peptide during the incubation of 10⁵ cell-equivalent lysates was 20000 c.p.m. for cells incubated for 10 min

Figure 1 GM-CSF-mediated priming of fMLP-induced O₂⁻ generation

Human neutrophils were incubated with or without fMLP, GM-CSF or PMA in cytochrome c-containing KR-Hepes according to the general protocol described in the Materials and methods section. (a) Shows O₂⁻ generation measured at 5 min after the addition of increasing concentrations of fMLP without (○) or with (●) 10 ng/ml of GM-CSF added 10 min before fMLP. In (b) is shown superoxide generation measured at 15 min after the addition of increasing concentrations of GM-CSF without (○) or with (●) 0.1 μ M fMLP added 10 min after GM-CSF. (c) Shows O₂⁻ generation measured at 5 min after the addition of increasing concentrations of PMA without (○) or with (●) 10 ng/ml of GM-CSF added 10 min before. Values are means $\pm$ S.E.M. for three or four experiments for (a) and three experiments for (b) and (c).

Figure 2 GM-CSF-mediated priming of fMLP-induced PtdInsP₃ production

Human neutrophils were labelled with ³²P and incubated in KR-Hepes medium according to the general protocol described in the Materials and methods section. The phospholipid fraction extracted from lysates of cells incubated with fMLP or its vehicle for 30 s was submitted to TLC to separate PtdInsP₃. (a) Autoradiogram of the TLC plate in which the position of PtdInsP₃ is indicated by an arrow, for cells incubated with increasing concentrations of fMLP with (+) or without (−) 10 ng/ml of GM-CSF as shown at the bottom. Similar experiments were repeated three times, and the ³²P content of PtdInsP₃ spots is plotted against concentrations of fMLP with (●) or without (○) GM-CSF in (b). In (c) the incorporation of ³²P into PtdInsP₃ was measured with cells incubated with increasing concentrations of GM-CSF added 10 min before vehicle (○) or 1 μM fMLP (●). Each point in (b) and (c) represents the percentage of control (the value without any addition) and results are means ± S.E.M. for triplicate experiments. For some points the error bar is within the diameter of the symbol.

without addition of PMA. Only 2–7% of the activity was found in membrane fractions. When cells were incubated with 0.1 μM PMA, the radioactivity increased to 30000, 39% of which was found in membrane fractions. Thus exposure of human neutrophils to PMA caused sustained activation of protein kinase C.

Activation of PI3K is responsible for fMLP-induced respiratory burst [21]. Addition of fMLP into ³²P-labelled human neutrophils resulted in production of the labelled PtdInsP₃, the product of PI3K, within 30 s in a concentration-dependent manner (Figure 2a). The GM-CSF-mediated priming was observed for fMLP-induced increases in PtdInsP₃ as well as in a concentration-dependent manner similar to that for the respiratory burst (compare Figures 2b and 2c with Figures 1a and 1b). PMA did not increase PtdInsP₃ production at all under any conditions tested (results not shown).

The membrane NADPH oxidase responsible for the phagocyte respiratory burst forms, upon activation, a large complex as a result of translocation of cytosolic factors, including several phox proteins such as p47^{phox} and p67^{phox} (see [22] for a review). The translocation is triggered by phosphorylation of these proteins on serine residues. ³²P was detected in the 47 kDa protein band in anti-p47^{phox} immunoprecipitates prepared from ³²P-labelled cell lysates (Figure 3). The ³²P content increased progressively when neutrophils were stimulated for 30 s by increasing concentrations of fMLP. This action of fMLP was also primed by GM-CSF in a concentration-dependent manner (Figures 3a and 3b). Activation of protein kinase C by adding PMA to cells gave rise to enormous phosphorylation of p47^{phox} without being primed by GM-CSF (results not shown).

Thus the data presented in Figures 1–3 lend a strong support to the notion that GM-CSF primes fMLP-induced PI3K activation leading to synergistic generation of O₂[−] via phosphorylation of p47^{phox}. Protein kinase C activated by

phorbol esters is responsible for the respiratory burst via a signalling pathway insensitive to the GM-CSF priming.

Selective effects of inhibitors and pertussis toxin on GM-CSF priming

Wortmannin added to human neutrophils 10 min before receptor stimulation gave rise to inhibition of the receptor-mediated respiratory burst as well as PI3K activation and p47^{phox} phosphorylation (Figure 4). The action of wortmannin shown in Figure 4 was mostly mimicked by LY294002, another inhibitor of PI3K (results not shown). Not only the response to fMLP alone, but also the responses to combined addition of GM-CSF with fMLP, were inhibited by wortmannin. The inhibition was dependent on the concentration of wortmannin and was almost complete at concentrations over 100 nM, with the half-maximally inhibitory concentration being less than 10 nM. As expected, neither the respiratory burst nor p47^{phox} phosphorylation was inhibited by wortmannin if the responses were induced by exposure of cells to PMA, which does not activate PI3K (Figure 4). Thus PI3K activation appeared to be essential for the respiratory burst to be triggered by receptor stimulation via p47^{phox} phosphorylation. More direct phosphorylation of p47^{phox} by PMA-activated protein kinase C by-passed PI3K activation.

Phosphorylation of p47^{phox} was not inhibited by genistein, an inhibitor of protein tyrosine kinase, regardless of whether the phosphorylation was provoked by fMLP/GM-CSF or PMA (Figure 5a). The results are not at variance with phosphorylation of p47^{phox} on serine residues. In contrast, GF109203X, an inhibitor of protein kinase C [23], inhibited phosphorylation of p47^{phox} caused by fMLP/GM-CSF, as well as by PMA, over seemingly the same inhibitory concentration (1–10 μM) range

Figure 3 GM-CSF-mediated priming of fMLP-induced p47^{phox} phosphorylation

Human neutrophils were labelled with ^{32}P , treated with di-isopropyl fluorophosphate and incubated in KR-Hepes medium according to the general protocol described in the Materials and methods section. The lysate prepared from the cells after 30 s incubation with fMLP were subjected to immunoprecipitation with anti-p47^{phox} antibody, SDS/PAGE and image analysing. (a) Shows autoradiograms with p47^{phox} protein bands indicated by arrows. Cells were incubated with increasing concentrations of fMLP with (+) or without (–) 10 ng/ml of GM-CSF added 10 min before. Experiments were repeated three times, and the radioactivities of the p47^{phox} bands are plotted in (b) as percentages of control (the value obtained without any addition). The values are means $\pm$ S.E.M. for three experiments. The effects of fMLP alone (○) and those with GM-CSF (●) are shown as a function of fMLP concentrations.

Figure 4 Inhibition by wortmannin of cell responses to fMLP under GM-CSF-primed or unprimed conditions

Human neutrophils, ^{32}P -labelled or not, were incubated with the additions of various concentrations of wortmannin, 0.1 μM fMLP, 10 ng/ml of GM-CSF or 0.1 μM PMA and subjected to analysis of respiratory burst (a), PtdInsP₃ production (b) or p47^{phox} phosphorylation (c) according to the general protocol described in the Materials and methods section. The responses to fMLP (○) or PMA (■) of unprimed cells or the responses to fMLP of GM-CSF-primed cells (●) are plotted against wortmannin concentrations. The values are means $\pm$ S.E.M. for triplicate experiments. Percentages of the highest values obtained with fMLP under the GM-CSF-primed conditions are plotted in (b) and (c).

Figure 5 Effects of protein kinase inhibitors on fMLP- or PMA-induced p47^{phox} phosphorylation and respiratory burst in GM-CSF-primed or unprimed cells

Human neutrophils, ^{32}P -labelled or not, were incubated according to the general protocol described in the Materials and methods section and were subjected to analyses of p47^{phox} phosphorylation (a) or O₂⁻ generation (b). The concentrations of fMLP, PMA and GM-CSF in the incubation medium were 0.1 μM , 0.1 μM and 10 ng/ml respectively and those of GF109203X and genistein are shown in (a) and (b). (a) Shows an autoradiogram of SDS/PAGE of ^{32}P -labelled cell lysates with p47^{phox} bands indicated by an arrow. In (b), O₂⁻ generation in response to fMLP (○), fMLP/GM-CSF (●) or PMA (■) is plotted against concentrations of GF109203X. The points represent means $\pm$ S.E.M. for three experiments, except for those for 10 and 100 nM inhibitor, which are single observations.

(Figure 5a). Such inhibitions of p47^{phox} phosphorylation were accompanied by inhibition of the respiratory burst induced by the same stimulants (Figure 5b). One might assume that fMLP/GM-CSF-induced activation of PI3K is responsible for activation of the GF109203X-susceptible protein kinase C that is directly responsible for phosphorylation of p47^{phox}.

The exposure of human neutrophils to pertussis toxin abolished fMLP-induced respiratory burst, PtdInsP₃ generation and p47^{phox} phosphorylation in either the absence or presence of GM-CSF without changes in the actions of GM-CSF alone (Table 1). As expected, the marked ability of PMA to cause respiratory burst and p47^{phox} phosphorylation was not at all inhibited upon pertussis toxin treatment of cells. An unexpected and exceptional

Table 1 Agonist- or PMA-induced O_2^- generation, PtdIns P_3 production and p47^{phox} phosphorylation in pertussis-toxin-treated cells

Human neutrophils were incubated for 2 h in RPMI-1640 medium with (+) or without (—) 100 ng/ml of pertussis toxin (PTX). ^{32}P labelling and incubation of these cells with GM-CSF (10 ng/ml), 0.1 μ M fMLP, and the latter two together or 0.1 μ M PMA were conducted according to the general protocol described in the Materials and methods section. The cell responses to these additions are expressed as percentage of the respective control value obtained without addition (which was 10.5 ± 2.8 nmol/5 min per 10^6 cells for O_2^- generation) and radioactivities (in c.p.m.) incorporated for PtdIns P_3 production and p47^{phox} phosphorylation. The 'control' obtained with pertussis-toxin-treated cells as percentage of the 'control' of the untreated cells is shown in parentheses. Results are means $\pm$ S.E.M. for triplicate experiments. Abbreviation: n.d.: not determined. * The effect of addition as compared with the respective control is significant ($P < 0.05$).

	PTX	Value (% of control)				
		Control	fMLP	GM-CSF	fMLP/GM-CSF	PMA
O_2^- generation	—	100 $\pm$ 27	220 $\pm$ 22*	175 $\pm$ 6*	436 $\pm$ 71*	667 $\pm$ 52*
	+	100 $\pm$ 26 (95.3)	91 $\pm$ 5	197 $\pm$ 16*	181 $\pm$ 7*	687 $\pm$ 30*
PtdIns P_3 production	—	100 $\pm$ 9	335 $\pm$ 11*	181 $\pm$ 19*	723 $\pm$ 47*	n.d.
	+	100 $\pm$ 9 (87.5)	92 $\pm$ 11	153 $\pm$ 7*	182 $\pm$ 8*	n.d.
p47 ^{phox} phosphorylation	—	100 $\pm$ 12	241 $\pm$ 15*	175 $\pm$ 13*	312 $\pm$ 28*	940 $\pm$ 115*
	+	100 $\pm$ 36 (78.4)	80 $\pm$ 29	121 $\pm$ 17	97 $\pm$ 20	1262 $\pm$ 196*

Figure 6 GM-CSF-mediated priming of fMLP-induced MAP kinase activation in the presence or absence of wortmannin

Human neutrophils were incubated according to the general protocol and subjected to measurement of MAP kinase activity as described in the Materials and methods section. The radioactivities incorporated into the ERK substrate peptide during 30 min incubation of the lysates from 10^5 cells are plotted against concentrations of fMLP added to unprimed ($\circ$) or 10 ng/ml GM-CSF-primed ($\bullet$) cells in (a) or against concentrations of GM-CSF used for priming in the absence ($\circ$) or presence ($\bullet$) of 0.1 μ M fMLP in (b). In (c) the MAP kinase activities obtained for cells incubated with 0.1 μ M fMLP plus 10 ng/ml GM-CSF ($\bullet$), 0.1 μ M fMLP alone ($\circ$) or 10 ng/ml GM-CSF alone ($\blacksquare$) were plotted against concentrations of wortmannin added into incubation medium 10 min before GM-CSF or its vehicle. Each point is the mean $\pm$ S.E.M. for three separate experiments.

result seen in Table 1 was the fact that GM-CSF-induced p47^{phox} phosphorylation was no longer observable in pertussis-toxin-treated cells. The possibility cannot be ruled out that variation in the data obtained from neutrophil preparations from different individuals might have obscured the small effect of GM-CSF on p47^{phox} phosphorylation.

GM-CSF priming of fMLP-induced activation of a MAP kinase that is not involved in the respiratory burst

MAP kinase (p42/44 ERK) activity increased when fMLP or GM-CSF was added to human neutrophils, in agreement with previous reports [24,25]. The combined addition activated MAP kinase considerably (Figures 6a and 6b). This GM-CSF priming of MAP kinase activation was more marked than the priming for the respiratory burst (compare Figure 1a with Figure 6a). The chemotactic peptide at a concentration as low as 1 nM (a

Figure 7 Effect of PD98059 on fMLP-induced O_2^- generation and MAP kinase activation in GM-CSF-primed neutrophils

Human neutrophils were incubated according to the general protocol in the presence of increasing concentrations of PD98059 (shown on the abscissa) and subjected to measurement of O_2^- generation ($\circ$) or MAP kinase activity ($\bullet$) as described in the Materials and methods section. The O_2^- generation and MAP kinase activity were 38.5 ± 2.6 nmol/5 min per 10^6 cells and 5649 ± 564 c.p.m. for 10^5 cells respectively in the absence of PD98059. The percentages of these control values (means $\pm$ S.E.M. for three experiments) are plotted against concentrations of PD98059.

concentration causing no significant respiratory burst either in the primed or unprimed cells) gave rise to 5-fold activation of MAP kinase in the GM-CSF-primed neutrophils (Figure 6a). The marked ability of fMLP to activate MAP kinase in GM-CSF-primed neutrophils was mediated by $G\beta\gamma$, since the action of the chemokine was lost when the primed cells had been exposed to pertussis toxin (results not shown).

Activation of MAP kinase by fMLP, GM-CSF or a combination of them was not totally inhibited by wortmannin (Figure 6c), suggesting that MAP kinase activation could not be responsible for fMLP-induced respiratory burst. In fact, addition

Figure 8 Accumulation of cAMP inhibits cellular responses to fMLP in smaller magnitudes in GM-CSF-primed cells than in unprimed cells

Human neutrophils, labelled with ³²P or not, were incubated with 50 μ M IBMX and increasing concentrations of prostaglandin E₁ added 3 min before 10 ng/ml of GM-CSF or its vehicle according to the general protocol described in the Materials and methods section. The incubated cells were subjected to analyses of cellular cAMP concentrations (a), respiratory burst (b) or PtdInsP₃ production (c). The responses to 0.1 μ M fMLP in unprimed (○) or GM-CSF-primed (●) cells or 0.1 μ M PMA in unprimed cells (□) are plotted against concentrations of prostaglandin E₁ (PGE₁). In (b) and (c), each point represents a percentage of the control value obtained without PGE₁. In (a) and (b), open and solid circles are means $\pm$ S.E.M. for three experiments. Open squares in (b) and all the points in (c) without error bars represent single observations.

of PD98059, an inhibitor of MAP kinase kinase [26], to the neutrophil suspension at concentrations from 1 to 30 μ M gave rise to a progressive decrease in the fMLP/GM-CSF-induced increase in the MAP kinase activity without detectable inhibition of the respiratory burst (Figure 7). Treatment of neutrophils with PMA also evoked MAP kinase activation, which, like other PMA effects, was not primed by GM-CSF (results not shown). PMA-induced activation of MAP kinase was totally inhibited by 10 μ M GF109203X, which inhibited the fMLP-induced activation only 30% in the GM-CSF-primed cells (results not shown).

Thus MAP kinase activation is unlikely to be responsible in itself for the respiratory burst to occur in neutrophils. Wortmannin-susceptible PI3K is unlikely to be a sole step at which $G\beta\gamma$ signals and GM-CSF signals converge to elicit priming, because the cytokine priming of MAP kinase activation

was still observed in wortmannin-treated cells (Figure 6c, the right-end points of plots).

Attenuation of cAMP-induced inhibition of fMLP actions in GM-CSF-primed neutrophils

The cAMP level within cells increased progressively when the concentration of prostaglandin E₁ was raised from 1 nM to 1 μ M during incubation of neutrophils in the medium fortified with 50 μ M IBMX, an inhibitor of cAMP phosphodiesterase. There was no significant difference in the extent of cAMP accumulation between GM-CSF-primed and unprimed cells (Figure 8a). In accordance with previous data [17], cAMP accumulation was accompanied by inhibitions of fMLP-induced O₂⁻ generation, but not by the PMA-induced one, (Figure 8b) and decreases in fMLP-induced PtdInsP₃ production (Figure 8c) in unprimed

Figure 9 Phosphorylation of cellular proteins on tyrosines in GM-CSF-treated cells

Human neutrophils, pertussis toxin (PTX)-treated (+) or not (—), were incubated according to the general protocol described in the Materials and methods section in (a). In (b), the incubation was conducted with various concentrations of GM-CSF alone for various lengths of time shown at the bottom. The cell lysates were submitted to immunoprecipitation (I.P.) with PY-20 (α PY) (a) or with anti-p85 (α p85) (b), followed by immunoblotting with rabbit polyclonal anti-phosphotyrosine antibodies. Two protein bands in (b) shown by arrows proved to be Shc in another experiment as described in the text.

cells. In GM-CSF-primed cells, however, cAMP was much less inhibitory to fMLP-induced respiratory burst and PI3K activation than in unprimed cells (Figures 8b and 8c). Thus GM-CSF-mediated priming of neutrophils not only enhanced the cellular responses to chemotactic receptor stimulation, but partly alleviated the inhibitory responses mediated by cAMP.

In other words, the GM-CSF-induced priming of neutrophil responses to fMLP was exaggerated upon accumulation of cAMP in cells. One could speculate that GM-CSF primes human neutrophils at just the same site on signalling pathways where cAMP or protein kinase A interacts to inhibit the signalling.

GM-CSF-induced protein tyrosine phosphorylation and association of certain signalling proteins

Exposure of human neutrophils to GM-CSF resulted in phosphorylation of cellular proteins on their tyrosine residues (Figure 9a). The ability of fMLP to cause tyrosine phosphorylation of cellular proteins was, if anything, much less than that of GM-CSF, and combination of fMLP with the cytokine did not significantly increase the cytokine-induced protein tyrosine phosphorylation. Nor was any detectable change in phosphorylation induced by pertussis-toxin treatment of cells to which fMLP was added (Figure 9a). In order to identify signalling proteins serving as the substrate of GM-CSF-activated tyrosine kinase(s), we prepared immunoprecipitates by adding to neutrophil lysates the antibodies raised against GM-CSF receptor β , SHPTP-2, Grb2 or Shc. The phosphotyrosine contents of these proteins increased progressively up to 10 min when the neutrophils were exposed to GM-CSF at concentrations higher than 1 ng/ml (results not shown). Association of the p85 regulatory subunit of PI3K with tyrosine-phosphorylated proteins [27,28] also occurred in response to GM-CSF in human neutrophils, as shown in Figure 9(b) in which the intensity of two p85-binding protein bands, shown by arrows, increased in time- and concentration-dependent manners in GM-CSF-treated cells. These two protein bands proved to be Shc in another experiment with the use of specific antibodies (results not shown). Neither tyrosine phosphorylation of these signalling proteins nor their association was affected by the addition of fMLP into the cell incubation medium (results not shown). The GM-CSF priming may target signalling cascades downstream of the step of protein tyrosine phosphorylation.

DISCUSSION

The GM-CSF-mediated priming of the responses of human neutrophil to fMLP was observed for activation of PI3K, phosphorylation of p47^{phox} and generation of O₂⁻ with the same fMLP concentration-dependency. The concentrations of GM-CSF required for the priming were essentially the same for these three parameters, which thus displayed good correlation between each other under these conditions studied. All of the responses to fMLP were abolished by prior exposure of primed or unprimed cells to pertussis toxin, indicating involvement of G $\beta\gamma$ liberated from fMLP receptor-coupled G_{i2}. Chemotactic-peptide-induced PtdInsP₃ production, p47^{phox} phosphorylation and respiratory burst were progressively inhibited by increasing concentrations of wortmannin with the same half-maximally inhibitory concentration less than 10 nM in either primed or unprimed cells. Wortmannin-sensitive PI3K should be thus upstream of the p47^{phox} phosphorylation that is directly responsible for activation of NADPH oxidase complex involved in

O₂⁻ generation. Addition of PMA to neutrophils did not activate PI3K, but caused marked p47^{phox} phosphorylation, leading to a considerable respiratory burst. The effects of PMA were neither primed by GM-CSF nor inhibited by wortmannin. In contrast, GF109203X, an inhibitor of protein kinase C, abolished not only PMA-induced but also fMLP-induced p47^{phox} phosphorylation and O₂⁻ generation. It would be likely, therefore, that fMLP-induced p47^{phox} phosphorylation is mediated by protein kinase C, which functions downstream of PI3K, as has been reported for various cell types, including haematopoietic cells [29–31].

Thus GM-CSF priming of the fMLP-induced respiratory burst appeared to be a reflection of synergistic activation of PI3K by G $\beta\gamma$ arising from fMLP receptor-coupled G_{i2} in the presence of protein(s) whose tyrosine residues have been phosphorylated via GM-CSF receptors. One could assume that synergistic activation of p110 β /p85 PI3K by G $\beta\gamma$ and tyrosine-phosphorylated peptides [11] could play a physiological role in cellular signal transduction.

GM-CSF also primed fMLP-induced activation of MAP kinase. The priming of MAP kinase suggests that the above-mentioned synergistic activation of PI3K is not the sole mechanism for GM-CSF-mediated priming of human neutrophils. PI3K is probably upstream of MAP kinase in G $\beta\gamma$ -initiated signalling pathways [32,33]. The activation of the MAP kinase signalling pathway, however, does not appear to play a crucial role in any aspect of NADPH oxidase activation because of the lack of correlation between MAP kinase activation and the respiratory burst at low concentrations of fMLP or in the presence of wortmannin, in agreement with previous reports [34–36]. O₂⁻ was generated in response to fMLP/GM-CSF in PD98059-treated cells in which no MAP kinase activation occurred. It should be noted that GM-CSF primed fMLP-induced MAP kinase activation, even in the presence of the highest concentration of wortmannin (the far right-hand points of plots in Figure 6c). It is thus reasonable to conclude that wortmannin-susceptible PI3K is not the sole site where GM-CSF signals interact with G $\beta\gamma$ -evoked signalling pathways to induce the priming. Seemingly, an additional site of the interaction would be located on signalling pathways originating from fMLP receptors and leading to MAP kinase activation by-passing PI3K.

It is tempting to speculate that the signalling site where increased cellular cAMP or thus activated protein kinase A interacts to inhibit the fMLP-induced respiratory burst would be very close to the GM-CSF priming site other than PI3K. We found that fMLP-induced activation of PI3K and generation of O₂⁻ were much less sensitive to the cAMP-induced inhibition in GM-CSF-primed cells than in unprimed cells. In other words, GM-CSF enhanced the neutrophil responses to fMLP more markedly in cells in which cAMP was accumulating than in control cells without the nucleotide accumulation. Increases in cellular cAMP concentrations have been reported to inhibit O₂⁻ generating responses of neutrophils to stimulation of G_i-coupled receptors such as those for fMLP [37] or C5a [38] as well as MAP kinase-activating responses of other cell types to stimulation of tyrosine kinase-related receptors [39–42] or G_i-coupled receptors [42]. Putting these findings together, protein kinase A activated by accumulating cAMP appears to interact with the first kinase of the MAP kinase cascades, Raf-1 or MEK kinase, or a step immediately upstream of Raf-1 and downstream of Ras. PI3K was also downstream of the site with which protein kinase A interacts to inhibit fMLP-induced respiratory burst [17]. Thus the GM-CSF priming site, in addition to PI3K, might be just the target of protein kinase A or could be localized immediately downstream of it.

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