British Journal of Pharmacology (1997) 122, 1135–1145

Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils

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1 The aim of this study was to establish the role of nitric oxide (NO) and cyclic GMP in chemotaxis and superoxide anion generation (SAG) by human neutrophils, by use of selective inhibitors of NO and cyclic GMP pathways. In addition, inhibition of neutrophil chemotaxis by NO releasing compounds and increases in neutrophil nitrate/nitrite and cyclic GMP levels were examined. The ultimate aim of this work was to resolve the paradox that NO both activates and inhibits human neutrophils.

2 A role for NO as a mediator of N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis was supported by the finding that the NO synthase (NOS) inhibitor L-NMMA (500 μM) inhibited chemotaxis; EC_{50} for fMLP 28.76 ± 5.62 and 41.13 ± 4.77 pmol/10^6 cells with and without L-NMMA, respectively. Similarly the NO scavenger carboxy-PTIO (100 μM) inhibited chemotaxis; EC_{50} for fMLP 19.71 ± 4.23 and 31.68 ± 8.50 pmol/10^6 cells with and without carboxy-PTIO, respectively.

3 A role for cyclic GMP as a mediator of chemotaxis was supported by the finding that the guanylyl cyclase inhibitor LY 83583 (100 μM) completely inhibited chemotaxis and suppressed the maximal response; EC_{50} for fMLP 32.53 ± 11.18 and 85.21 ± 15.14 pmol/10^6 cells with and without LY 83583, respectively. The same pattern of inhibition was observed with the G-kinase inhibitor KT 5823 (10 μM); EC_{50} for fMLP 32.16 ± 11.35 and >135 pmol/10^6 cells with and without KT 5823, respectively.

4 The phosphatase inhibitor, 2,3-diphosphoglyceric acid (DPG) (100 μM) which inhibits phospholipase D, attenuated fMLP-induced chemotaxis; EC_{50} for fMLP 19.15 ± 4.36 and 61.52 ± 16.2 pmol/10^6 cells with and without DPG, respectively.

5 Although the NOS inhibitors L-NMMA and L-canavanine (500 μM) failed to inhibit fMLP-induced SAG, carboxy-PTIO caused significant inhibition (EC_{50} for fMLP 36.15 ± 7.43 and 86.31 ± 14.06 nM and reduced the maximal response from 22.14 ± 1.5 to 9.8 ± 1.6 nmol O_2^-/10^6 cells/10 min with and without carboxy-PTIO, respectively). This suggests NO is a mediator of fMLP-induced SAG.

6 A role for cyclic GMP as a mediator of SAG was supported by the effects of G-kinase inhibitors KT 5823 (10 μM) and Rp-8-pCPT-cGMPS (100 μM) which inhibited SAG giving EC_{50} for fMLP of 36.26 ± 8.77 and 200.01 ± 43.26 nM with and without KT 5823, and 28.35 ± 10.8 and 49.25 ± 16.79 nM with and without Rp-8-pCTP-cGMPS.

7 The phosphatase inhibitor DPG (500 μM) inhibited SAG; EC_{50} for fMLP 33.93 ± 4.23 and 61.12 ± 14.43 nM with and without DPG, respectively.

8 The NO releasing compounds inhibited fMLP-induced chemotaxis with a rank order of potency of GEA 3162 ($IC_{50} = 14.72 \pm 1.6$ μM) > GEA 5024 ($IC_{50} = 18.44 \pm 0.43$ μM) > SIN-1 ($IC_{50} > 1000$ μM). This order of potency correlated with their ability to increase cyclic GMP levels rather than the release of NO, where SIN-1 was most effective (SIN-1 ($EC_{50} = 37.62 \pm 0.9$ μM) > GEA 3162 ($EC_{50} = 39.7 \pm 0.53$ μM) > GEA 5024 ($EC_{50} = 89.86 \pm 1.62$ μM)).

9 In conclusion, chemotaxis and SAG induced by fMLP can be attenuated by inhibitors of phospholipase D, NO and cyclic GMP, suggesting a role for these agents in neutrophil activation. However, the increases in cyclic GMP and NO induced by fMLP, which are associated with neutrophil activation, are very small. In contrast much larger increases in NO and cyclic GMP, as observed with NO releasing compounds, inhibit chemotaxis.

Keywords: Neutrophils; nitric oxide; superoxide anion; chemotaxis; guanylyl cyclase; cyclic GMP; G-kinase; phospholipase D; nitric oxide synthase

Introduction

Neutrophils represent the first line of host defence against bacterial infection. They are recruited from the bloodstream by chemotactic factors generated and released locally in injured tissue (Barten *et al*., 1976; Weiss, 1989). Once at the site of inflammation, neutrophils release toxic substances such as superoxide anion (O_2^-) and lysosomal enzymes, and ingest micro-organisms by phagocytosis.

Neutrophil locomotion to a specific chemoattractant is a complex, multi-step process requiring ligation of a cell surface receptor, transduction of a signal from the receptor to intracellular effectors, reorganisation of the cytoskeleton and finally a directed crawling movement towards the source of chemotaxin (Cassimeris & Zigmond, 1990). Several cellular pathways, as well as numerous specific macromolecules have been identified as being essential for the process of neutrophil movement (Cassimeris & Zigmond, 1990; Gaudry *et al*., 1992; Amatruda *et al*., 1993). However, the effector signalling pathways activated in neutrophils to promote chemotaxis are still poorly understood. Unstimulated and primed human and rat neutrophils have been shown to generate and release factors with the pharmacological characteristics of nitric oxide (NO) (Stephens & Snyderman, 1982; Rimele *et al*., 1988; 1991; Wright *et al*., 1989; Schmidt *et al*., 1989; Salvemini *et al*., 1989; McCall *et al*., 1989; Myers *et al*., 1990; Mehta *et al*., 1990; Lee

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et al., 1990; Yui *et al*., 1991; Moncada & Higgs, 1991; Moncada *et al*., 1991; Lopez Farre *et al*., 1991; Kadota *et al*., 1991). The release of NO is regulated by nitric oxide synthase (NOS), a cytosolic enzyme that catalyses the conversion of L-arginine to L-citrulline and NO (Moncada *et al*., 1991). Through the stimulation of guanylyl cyclase, nitric oxide increases guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation. Intracellular accumulation of cyclic GMP has been suggested to regulate neutrophil chemotaxis *in vitro* (Sandler *et al*., 1975; Smith & Ignarro, 1975; Stephens & Snyderman, 1982; Anderson *et al*., 1986; Kaplan *et al*., 1989). Consistent with these concepts, it has been shown that fMLP-mediated chemotaxis was decreased by an inhibitor of NOS, N^G-monomethyl-L-arginine (L-NMMA), and that exogenous cyclic GMP reversed this inhibition (Kaplan *et al*., 1989). Furthermore, it has been shown that NOS inhibitors significantly attenuate chemotaxis of unstimulated and primed human neutrophils *in vitro* and that these effects were specific and modulated by cyclic GMP (Belenky *et al*., 1993). These two latter experiments suggest a role for NO as an intracellular messenger mediating neutrophil chemotaxis, possibly by increasing cell polarization (Caterina & Deureotes, 1991). This is supported by the recent demonstration that exogenous NO induced chemotaxis of neutrophils *in vitro* (Beauvais *et al*., 1995).

However, in contradiction to this, there is also data suggesting that NO or NO-releasing compounds can inhibit aspects of neutrophil activation such as chemotaxis, degranulation, leukotriene (LT) production and O_2^- release (Clancy *et al*., 1982). Some of these effects were suggested to be mediated, at least in part, by an increase of cyclic GMP from activation of soluble guanylyl cyclase (Ney *et al*., 1990; Schroder *et al*., 1990; Kubes *et al*., 1991; Wenzel-Seifert *et al*., 1991; Moilanen *et al*., 1993).

The aim of this study was to clarify the role of NO and cyclic GMP in chemotaxis and superoxide anion generation (SAG) by human neutrophils. For this purpose, the inhibitory effects of the NOS inhibitors, L-NMMA and L-canavanine; the NO scavenger carboxy-PTIO; the guanylyl cyclase inhibitor LY 83583; the G-kinase inhibitors, KT 5823 and Rp-8-cCPT-cGMPS and the phosphatase inhibitor, 2,3 diphosphoglycerate (DPG) have been investigated. In addition the NO-releasing compounds, 3-morpholinosydnonimine (SIN-1) and 4-aryl-substituted oxatriazol derivatives (GEA 3162 and GEA 5024) have been tested for inhibition of neutrophil chemotaxis as well as for their ability to increase neutrophil nitrate/nitrite and cyclic GMP levels. The ultimate aim of this work was to resolve the paradox that NO appears to be able to both activate and inhibit human neutrophils.

Methods

Isolation of human neutrophils

Human neutrophils were isolated as described previously (Talpain *et al*., 1995). Any contaminating red cells were removed by hypotonic lysis with ice-cold NaCl (0.2% w/v) and the cells returned to isotonic conditions with NaCl (1.6%). The cells were > 95% viable as determined by trypan blue exclusion and were resuspended as required below.

Chemotaxis procedure

Cells were resuspended at a concentration of 3×10^6 cells ml^{-1} in RPMI 1640 medium (HEPES buffered, without glutamine, Gibco) and chemotaxis measured in a 96 well chemotaxis chamber (Neuroprobe, Cabin John, Md). The bottom wells of the chamber were filled with chemoattractant, N-formyl-methionyl-lencyl-phenylalanine (fMLP; 0.1 – 3 μM) in 30 μl RPMI medium which had been warmed to 37°C. The top plate with the filter (3 μm) installed was then inverted onto the filled bottom plate, and the upper wells filled with cells (225 μl) which had been treated with inhibitor or RPMI medium. In the case of L-NMMA, SIN-1, GEA 3162 and GEA 5024, the cells were pre-incubated with these drugs for 10 – 45 min, as appropriate. With carboxy-PTIO, LY 83583, KT 5823 and Rp-8-pCPT-cGMPS, no preincubation was required. The chamber was then incubated for 45 min at 37°C in a moist, 5% CO_2 atmosphere. At the end of the incubation period, the filter was removed, washed, fixed and stained with Diff Quick (Baxter Diagnostics AG; fixative-fast green in methanol for 5 min, eosin G in phosphate buffer for 5 min, thiazine dye in phosphate buffer for 5 min). Chemotaxis was quantified spectrophotometrically by measuring absorbance at 550 nm and the magnitude of the absorbance taken as directly proportional to the number of cells which have migrated and are trapped in the filter. Basal absorbance was taken as cells without fMLP. Each incubation was carried out in triplicate and the values were averaged.

Superoxide anion generation

Neutrophil SAG was assayed by spectrophotometric evaluation of the reduction of ferricytochrome C to ferrocytochrome C (A 550 nm) as described previously (Armstrong, 1995). Briefly, cells (1.5×10^6 cells ml^{-1}) were resuspended in PBS containing cytochrome C (2.5 mg ml^{-1}) and cytochalasin B (5 μg ml^{-1}). Cells were treated with PBS or inhibitor, immediately before the addition to the tubes containing fMLP (3 – 300 nM) and incubated for 10 min at 37°C. With L-NMMA, L-canavanine and DPG, cells were pre-incubated for 10 – 45 min, as appropriate. The reaction was terminated by immersing the tubes in ice for 5 min and the samples were centrifuged at 300 *g*, at 4°C for 10 min, to sediment the cells. Aliquots (200 μl) from each tube were dispensed into a 96 well plate and the absorbance at 550 nm was measured. Basal absorbance was taken as cells without fMLP. Each incubation was carried out in triplicate and the values were averaged.

Cyclic GMP measurement

Neutrophils were resuspended to give 5×10^6 cells ml^{-1} in PBS containing 0.25 mM isobutylmethylxanthine (IBMX). Cells (450 μl) were incubated for 10 min at 37°C, with buffer, fMLP or NO releasing compounds, and the reaction stopped by the addition of ethanol (1.0 ml). Five minutes later, the samples were centrifuged at 650 *g*, 20°C for 20 min, the ethanolic supernatants removed and evaporated to dryness at 55°C. The residue was dissolved in assay buffer (0.5 ml) and centrifuged at 1900 *g*, 4°C, for 30 min to remove insoluble material. Two samples (50 μl) of the supernatant were assayed. Both samples and standards were acetylated with a mixture of triethylamine/acetic anhydride (2:1) before measurement of cyclic GMP by radioimmunoassay. Each assay tube contained the following substances: unlabelled cyclic GMP (Sigma) (50 μl of 0.0625 – 32 nM) or samples to be measured and sodium acetate buffer (pH 6.0) (200 μl of 0.05 mM) containing specific antibody and guanosine 3′,5′-cyclic phosphoric acid 2′-O′-succinyl-3-[^{125}I]-iodotyrosine methyl ester (5000 c.p.m./tube, Amersham). The assay tubes were kept at 4°C for 1 h, then donkey anti-rabbit serum (50 μl of 1:10 dilution in phosphate buffer) and normal rabbit serum (50 μl of 1:100 dilution in phosphate buffer) were added and the assay tubes were incubated overnight. The assay tubes were washed with 6% polyethylene glycol in deionized water (1.0 ml) and then centrifuged at 1900 *g* for 30 min at 4°C. The supernatant was aspirated and the residue counted with a gamma counter.

Total nitrate and nitrite production

Total nitrate and nitrite production was measured by use of Cayman's nitrate/nitrite assay kit (Alexis Corporation). The measurement is a simple two-step process where nitrate is converted to nitrite with nitrate reductase, and nitrite is measured spectrophotometrically at 540 nm by use of the Griess reagents.

Cells (1.5×10^6 cells ml^{-1}) were resuspended in PBS and treated with NO-releasing compounds (GEA 3162, GEA 5024 and SIN-1) for 10 min at 37°C before addition to the tubes containing PBS and incubated for 30 min at 37°C. With fMLP, cells were treated with PBS for 10 min at 37°C before addition to the tubes containing fMLP (3–300 nM) and incubated for 30 min at 37°C. The reaction was terminated by immersing the tubes in ice for 5 min and the samples were centrifuged at 300 *g*, at 4°C for 10 min to sediment the cells. Aliquots (80 μl) were dispensed into a microtitre plate and assayed spectrophotometrically.

Data analysis

Chemotaxis and SAG EC_{50} values were calculated as the concentration of fMLP required to produce 50% of the maximal response obtained in each experiment with fMLP (300 nM). As such, when drug treatment suppressed the fMLP maximum response, this observed maximum (i.e. determined in the presence of drug) was used for the purpose of determining the EC_{50} value. Each concentration-effect curve was illustrated by use of the Apple Macintosh programme 'Kaleidagraph' and the EC_{50} value determined.

Effects of the NO-releasing compounds, GEA 3162, GEA 5024 and SIN-1, on fMLP-induced chemotaxis were expressed as the percentage inhibition of the response produced by a submaximally effective concentration of fMLP (100 nM). The EC_{50} values for GEA 5024 and SIN-1 were determined relative to the maximum effect achieved with GEA 3162. From EC_{50} values, equieffective concentration-ratios (EEC) were calculated relative to the standard inhibitor, GEA 3162 (EEC = 1).

Statistical analysis

Data are expressed as the mean ± s.e.mean, of the averaged result taken from a minimum of four separate experiments. Data were analysed with Student's paired two-tailed *t* test. In addition, data involving multiple comparisons were analysed by ANOVA (two factor with replication) by use of microsoft Excel. A value of $P < 0.05$ was taken as significant.

Materials

The following compounds were gifts which are gratefully acknowledged: 4-aryl-substituted oxatriazol derivatives GEA 3162 (3-(3′,4′-dichlorophenyl)-1,2,3,4-oxatriazol-5-imine) and GEA 5024 (3-(3′-chloro-2′-methylphenyl)-1,2,3,4-oxatriazol-5-imine) from Dr S.B. Pedersoen (GEA Ltd, Copenhagen, Denmark) specific antibody against acetylated cyclic GMP from Dr I. Gow (Department of Physiology, University of Edinburgh).

N-formyl-methionyl-leucyl-phenylalanine (fMLP), L-canavanine, PBS (containing Ca^{2+} and Mg^{2+}), 2,3-diphosphoglycerate (DPG), trypan blue, guanosine 3′:5′-cyclic monophosphate, polyethylene glycol, cytochrome C and cytochalasin B were purchased from Sigma; RPMI 1640 from Gibco; N^G-monomethyl-L-arginine (L-NMMA), 6-anilinoquinoline-5-8-quinone (LY 83583), (8**R**,9**S**,11**S**)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b, 11a-triazadibenzo (a,g) cycloocta (cde)-trinden-1-one (KT 5823) from Calbiochem; Rp-8-(4-chlorophenylthio)-guanosine-3′-5′-cyclic monophosphorothioate (Rp-8pCPT-cGMPS) from Biolog; Diff-Quick from Gamidor; 2-(4-carboxyphenyl)-4,4,5,5-tetra methylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and 3-morpholinosydnonimine (SIN-1) from Tocris Cookson; Cayman's nitrate/nitrite assay kit from Alexis Corporation; quanosine 3′,5′-cyclic phosphoric acid, 2′-O′-succinyl-3-[^{125}I]-iodo tyrosine methyl ester from Amersham; triethylamine and acetic anhydride from BDH. Donkey-anti-rabbit serum and normal rabbit serum were supplied by the Scottish Antibody Production Unit (Carluke).

Results

Neutrophil chemotaxis

Effect of NOS inhibition When neutrophils were preincubated with the NOS inhibitor, L-NMMA (500 μM) for 45 min at 37°C, significant attenuation ($P < 0.001$, ANOVA) of fMLP-induced neutrophil chemotaxis occurred (Figure 1a), EC_{50} for fMLP 28.76 ± 5.62 and 41.13 ± 4.77 pmol/10^6 cells, $n = 5$ ($P < 0.05$) in the absence and presence of L-NMMA, respectively. The maximum effect of fMLP was reduced from 1.1 ± 0.09 to 0.72 ± 0.09, $n = 5$ ($P < 0.05$). Similarly, L-NMMA at a concentration of 100 μM induced a significant but less

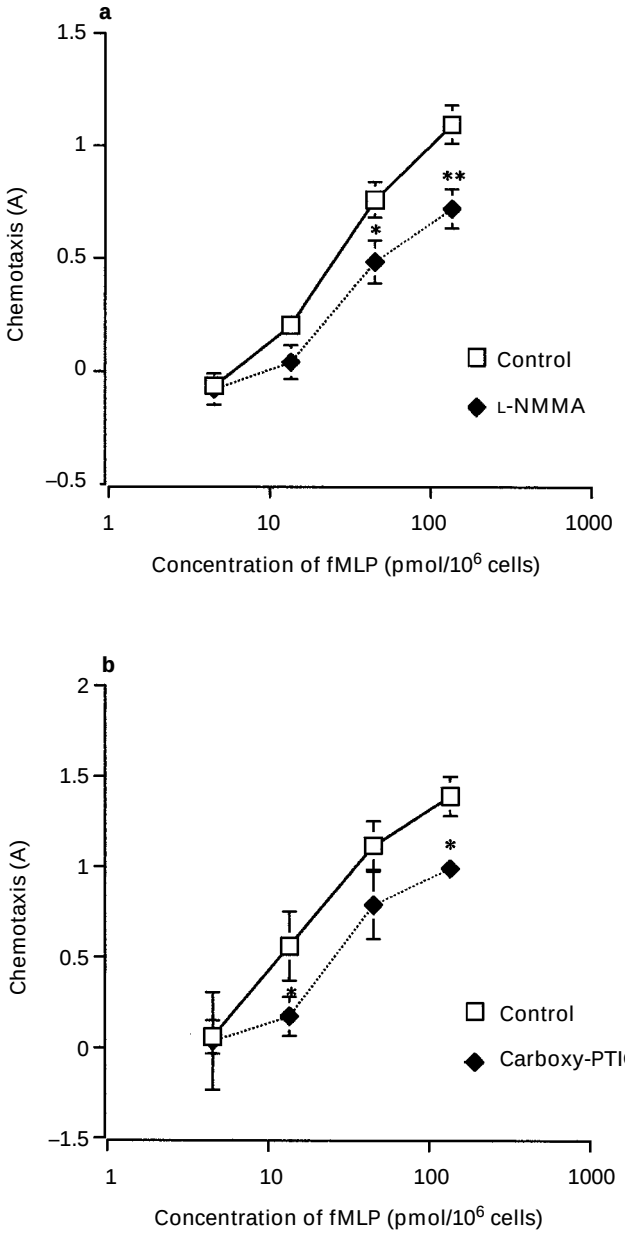

Figure 1 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with (a) L-NMMA (500 μM) and (b) carboxy-PTIO (100 μM). Cells were preincubated with L-NMMA for 45 min at 37°C. No preincubation was required with carboxy-PTIO. The values are the mean, and vertical lines show s.e.mean, of 5 different donors. Statistically significant difference of $*P < 0.05$ and $**P < 0.01$.

pronounced attenuation of neutrophil chemotaxis (data not shown; $P<0.05$).

Effect of a NO scavenger Carboxy-PTIO, a NO scavenger, at a concentration of 100 μM caused slight attenuation ($P<0.05$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 1b); EC_{50} for fMLP 19.71 ± 4.23 and 31.68 ± 8.50 pmol/10^6 cells, $n=4$, ($P=0.052$) in the absence and presence of carboxy-PTIO, respectively.

Effect of guanylyl cyclase inhibition LY 83583, an inhibitor of guanylyl cyclase, at concentrations of 10 μM ($P<0.01$, ANOVA) and 100 μM ($P<0.001$, ANOVA) caused significant attenuation of fMLP-induced neutrophil chemotaxis (Figure 2a and b); EC_{50} for fMLP 19.07 ± 4.3 and 47.04 ± 7.52, $n=4$ ($P<0.05$) and 32.53 ± 11.18 and >135 pmol/10^6 cells, $n=4$ ($P<0.05$) in the absence and presence of LY-83583 at the two concentrations, respectively. LY 83583 at a concentration of 100 μM caused a significant reduction in maximal effect of fMLP from 1.65 ± 0.01 to 0.32 ± 0.05, $n=4$ ($P<0.005$).

Effect of G-kinase inhibition KT 5823, a specific inhibitor of cyclic GMP-dependent protein kinase (G-kinase) at a concentration of 1 μM had no significant inhibitory effect ($P>0.05$, ANOVA) on fMLP-induced neutrophil chemotaxis (Figure 3a); EC_{50} for fMLP 19.07 ± 4.29 and 35.45 ± 13.54 pmol/10^6 cells, $n=4$ ($P>0.05$) in its absence and presence, respectively. However, at a concentration of 10 μM, KT 5823 completely inhibited ($P<0.001$, ANOVA) fMLP-induced chemotaxis (Figure 3b); EC_{50} for fMLP 32.16 ± 11.35 and >135 pmol/10^6 cells, $n=4$ ($P<0.005$) in the absence and presence of KT 5823, respectively.

Rp-8-pCPT-cGMPS, another inhibitor of cyclic GMP-dependent protein kinase G1α both at concentrations of 10 and 100 μM had no significant inhibitory effect ($P>0.05$, ANOVA) on fMLP-induced chemotaxis (figures not shown); EC_{50} for fMLP 19.07 ± 4.3 and 32.67 ± 14.81, $n=4$ ($P>0.05$) and 32.16 ± 11.35 and 21.67 ± 4.15 pmol/10^6 cells, $n=4$ ($P>0.05$) in its absence and presence at the two concentrations, respectively.

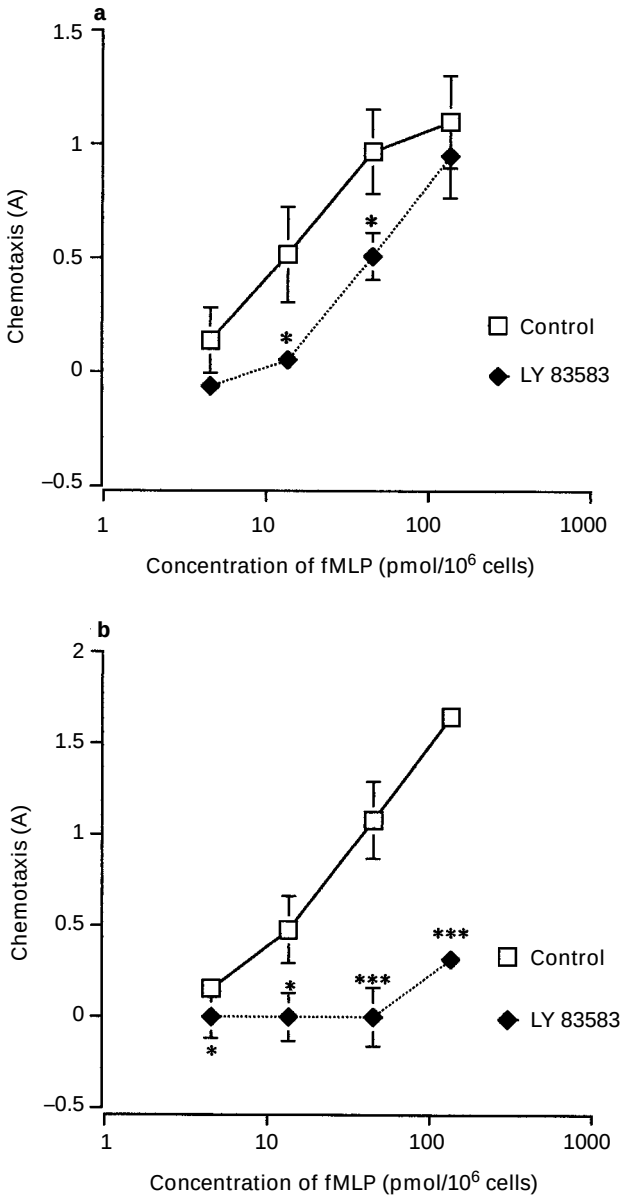

Figure 2 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with LY 83583 (a) 10 μM and (b) 100 μM. The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $*P<0.05$ and $***P<0.005$.

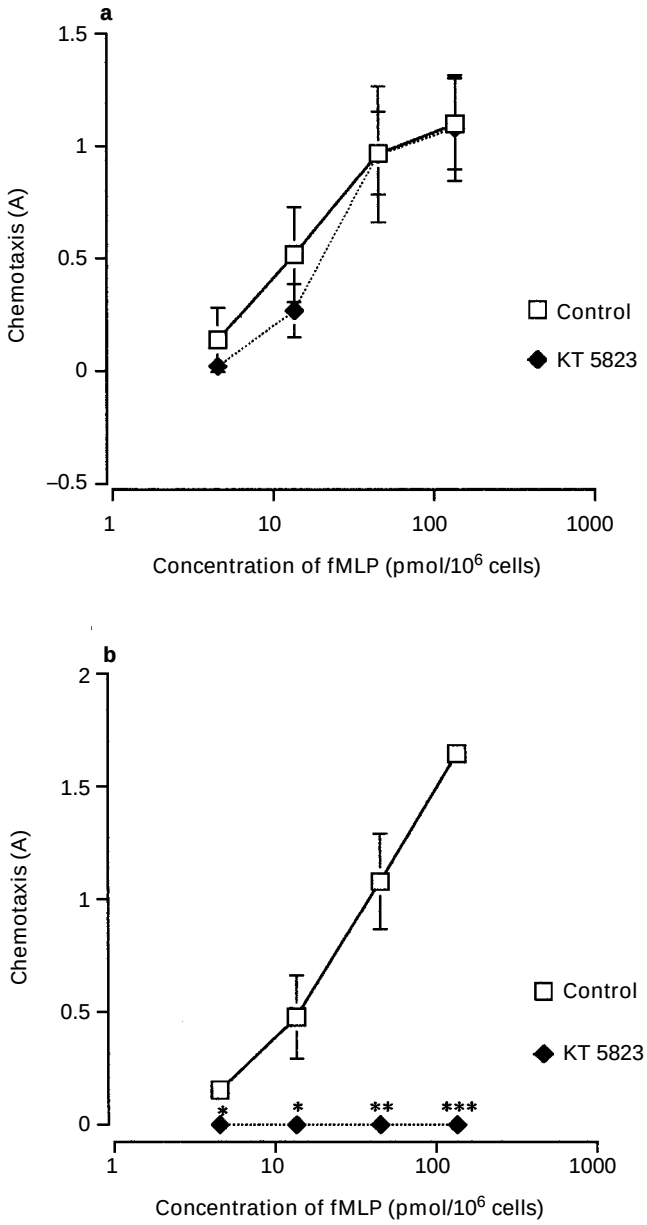

Figure 3 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with KT 5823 (a) 1 μM and (b) 10 μM. The values are the mean, and vertical lines show s.e.mean, of 5 different donors. Statistically significant difference, $*P<0.05$, $**P<0.01$ and $***P<0.005$.

Effect of phosphatase inhibition DPG, an inhibitor of inositol polyphosphate-5-phosphatase, at concentrations of 10 and 100 μM (both $P<0.001$, ANOVA) caused significant attenuation of fMLP-induced neutrophil chemotaxis (Figure 4a and b); EC_{50} for fMLP 25.97 ± 4.25 and 34.37 ± 4.04, $n=4$ ($P<0.05$) and 19.15 ± 4.36 and 61.52 ± 16.2 pmol/10^6 cells, $n=4$ ($P<0.05$) in its absence and presence at the two concentrations, respectively. Maximal effects of fMLP were reduced by 10 and 100 μM DPG from 1.38 ± 0.05 to 0.86 ± 0.14 and from 1.39 ± 0.11 to 0.72 ± 0.14, $n=4$ ($P<0.05$), respectively.

Neutrophil superoxide anion generation (SAG)

Effect of NOS inhibition When neutrophils were preincubated with L-NMMA at a concentration of 100 μM for 45 min at 37°C, L-NMMA caused no significant inhibition ($P<0.05$, ANOVA) of fMLP-induced SAG in human neutrophils (figure not shown); EC_{50} for fMLP 54.24 ± 11.5 and 61.36 ± 12.93 nM, $n=6$ ($P>0.05$) in its absence and presence, respectively. Even when the concentration of L-NMMA was increased to 500 μM, no significant inhibition of fMLP-induced SAG was observed (EC_{50} for fMLP 48.93 ± 12.51 and 57.13 ± 10.93 nM, respectively $n=6$ ($P>0.05$)).

Similarly, L-canavanine at concentrations of 100 and 500 μM caused no significant inhibition (both $P<0.05$, ANOVA) of fMLP-induced SAG in human neutrophils (figure not shown); EC_{50} for fMLP 36.75 ± 7.87 and 32.9 ± 5.41, $n=5$ ($P>0.05$) and 36.75 ± 7.87 and 33.60 ± 8.28 nM, $n=5$ ($P>0.05$) in its absence and presence at the two concentrations, respectively.

Effect of a NO scavenger Carboxy-PTIO, a NO scavenger, at a concentration of 100 μM caused significant attenuation ($P<0.05$, ANOVA) of fMLP-induced SAG in human neutrophils (Figure 5); EC_{50} for fMLP 36.15 ± 7.43 and 86.31 ± 14.06 nM, $n=6$ ($P<0.05$) in its absence and presence, respectively. Maximal effects of fMLP were reduced from 22.14 ± 1.5 to 9.8 ± 1.6 nmol O_2^-/10^6 cells 10 min^{-1} at 300 nM.

Effect of guanylyl cyclase inhibition LY 83583, an inhibitor of guanylyl cyclase, at concentrations of 10 and 100 μM caused no significant inhibition of fMLP-induced SAG in human neutrophils (figure not shown); EC_{50} for fMLP 23.81 ± 1.76 and 18.96 ± 4.52, $n=4$ ($P>0.05$), and 26.27 ± 1.44 and 13.73 ± 3.33 nM, $n=4$ ($P>0.05$) in its absence and presence at the two concentrations, respectively. However, a significant enhancement in fMLP-induced SAG at the lowest concentrations of fMLP tested (3–10 nM) was observed with 100 μM LY 83583 ($P<0.05$).

Effect of G-kinase inhibition KT 5823, a specific inhibitor of G-kinase, at concentrations of 1 and 10 μM caused significant inhibition (both $P<0.001$, ANOVA) of fMLP-induced SAG in human neutrophils (Figure 6a and b); EC_{50} for fMLP 34.28 ± 8.9 and 52.59 ± 4.9, $n=5$ ($P=0.05$) and 36.26 ± 8.77 and >300 nM, $n=5$ ($P<0.05$) in its absence and presence at the two concentrations, respectively. Maximal effects of fMLP were reduced by 1 and 10 μM KT 5823 from 22.22 ± 0.68 to 12.17 ± 1.43, $n=5$, ($P<0.005$) and from 28.64 ± 4.15 to 6.59 ± 2.06 nmol O_2^-/10^6 cells/10 min at 300 nM, $n=5$ ($P<0.001$), respectively.

Rp-8-pCPT-cGMPS (100 μM), a moderately potent inhibitor of cyclic GMP-dependent protein kinase G1α, caused some

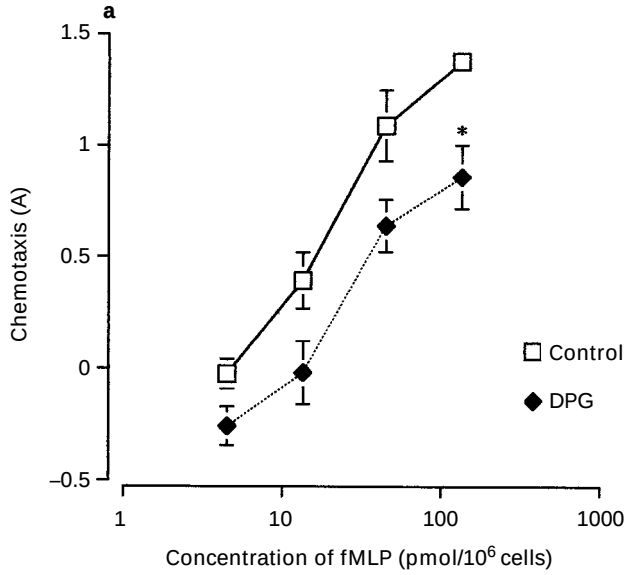

Figure 4 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with 2,3-diphosphoglyceric acid (DPG) (a) 10 μM and (b) 100 μM. The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $*P<0.05$.

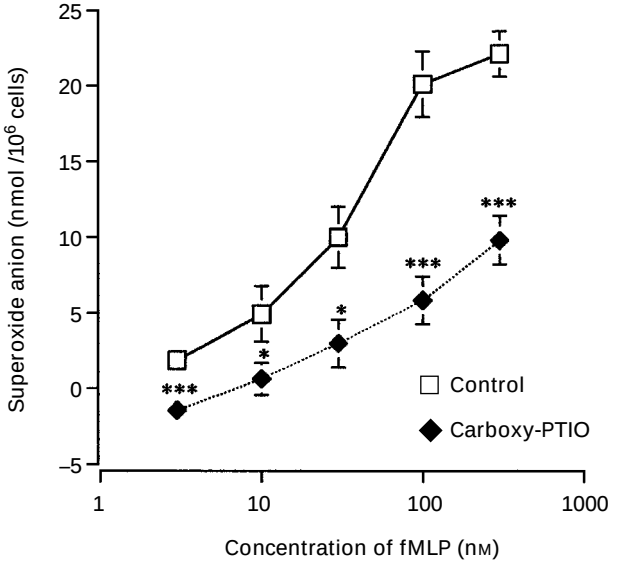

Figure 5 Log concentration-effect curves for fMLP induced superoxide anion generation in control cells and cells treated with carboxy-PTIO (100 μM). The values are the mean, and vertical lines show s.e.mean, of 6 different donors. Statistically significant difference, $*P<0.05$ and $***P<0.005$.

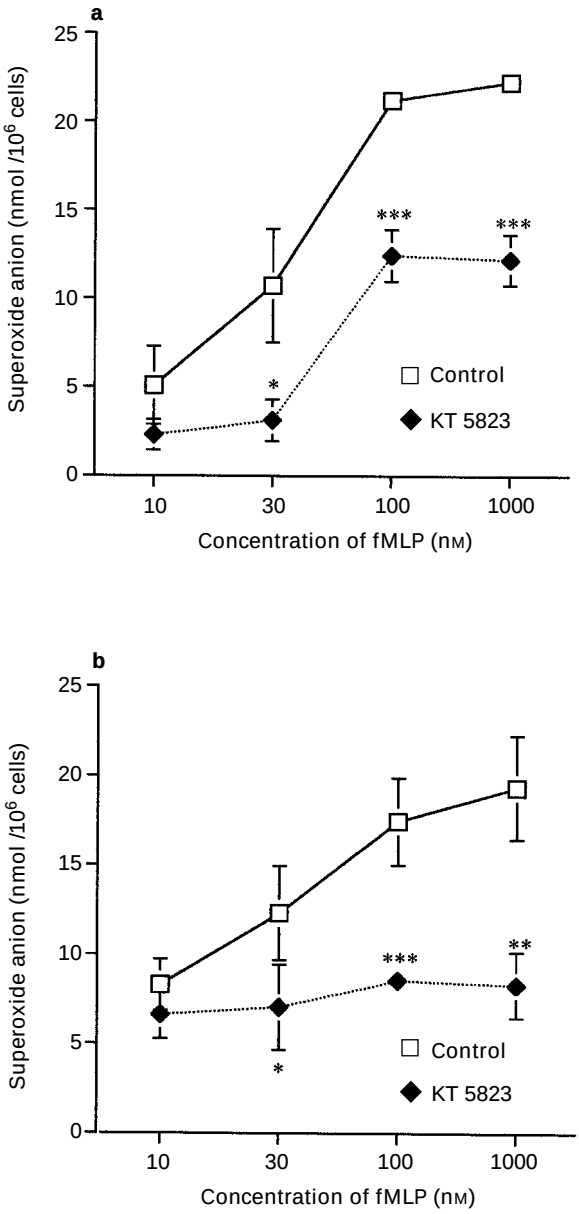

Figure 6 Log concentration-effect curves for fMLP-induced superoxide anion generation in control cells and cells treated with KT 5823 (a) 1 μM and (b) 10 μM. The values are the mean, and vertical lines show s.e.mean, of 5 different donors. Statistically significant difference, $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$.

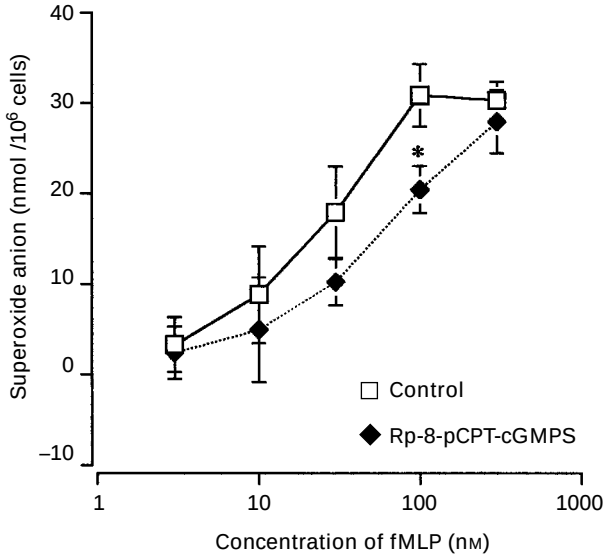

Figure 7 Log concentration-effect curves for fMLP-induced superoxide anion generation in control cells and cells treated with Rp-8-pCPT-cGMPS (100 μM). The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $^*P<0.05$.

inhibition ($P=0.058$, ANOVA) of fMLP-induced SAG in human neutrophils (Figure 7); EC_{50} for fMLP 28.35 ± 10.82 and 49.25 ± 16.79 nM, $n=4$ ($P<0.05$) in its absence and presence, respectively.

Effects of phosphatase inhibition DPG, an inhibitor of inositol polyphosphate-5-phosphatase, at a concentration of 500 ($P<0.005$, ANOVA) but not 100 μM caused significant inhibition of fMLP-induced SAG in human neutrophils (Figure 8a and b). EC_{50} for fMLP 36.23 ± 9.05 and 44.59 ± 8.88, $n=4$ ($P<0.05$, one-tailed test only) and 33.93 ± 4.23 and 61.12 ± 14.43 nM, $n=4$ ($P<0.05$) were obtained in the absence and presence of 100 and 500 μM DPG, respectively. Maximal effects of fMLP were reduced by 100 and 500 μM DPG from 25.64 ± 1.75 to 24.18 ± 2.15 ($n=4$) and from 26.17 ± 2.7 to 20.59 ± 3.0 nmol O_2^- 10^6 cells 10 min^{-1}, $n=4$ ($P<0.05$), respectively. In contrast, when neutrophils were preincubated with 100 and 500 μM DPG for 10 min at 37°C, neither concentration of DPG caused a significant effect on fMLP-induced SAG in human neutrophils (Figure 9a); EC_{50} for fMLP 27.32 ± 6.06 and 26.95 ± 6.31 nM, $n=4$ ($P>0.05$) and 25.57 ± 4.63 and 46.13 ± 23.05 nM, $n=4$ ($P>0.05$) in its absence and presence at the two concentrations, respectively. In addition, no significant effect on fMLP-induced SAG in human neutrophils was observed when neutrophils were preincubated with 100 and 500 μM DPG for 20 min at 37°C (Figure 9b); EC_{50} for fMLP 26.97 ± 2.32 and 30.23 ± 9.98 nM, $n=4$ ($P>0.05$) and 26.97 ± 2.31 and 26.90 ± 9.53 nM, $n=4$ ($P<0.05$) in its absence and presence at the two concentrations, respectively.

Effect of NO-releasing compounds on neutrophil chemotaxis

fMLP at a concentration of 100 nM induced a submaximal migration of neutrophils. When neutrophils were preincubated with NO-releasing compounds, GEA 3162 and GEA 5024, for 10 min at 37°C, these two compounds (1–100 μM) caused concentration-related inhibition of fMLP-induced chemotaxis (Figure 10), producing complete inhibition at a concentration of 100 μM ($IC_{50}=14.71\pm1.6$ μM, $n=5$ and 18.44 ± 0.43 μM, $n=5$, respectively). SIN-1 was a significantly ($P<0.05$) less potent inhibitor of fMLP-induced chemotaxis than GEA 3162; SIN-1 (1 mM induced a maximum inhibition of 24.99 ± 7.64% ($n=8$) (Figure 11). If the maximal effect of GEA 3162 at 100 μM was taken to be 100% inhibition, the IC_{50} for SIN-1 was >1000 μM ($n=8$), giving an EEC >62.7.

Effect of the NO-releasing compounds and fMLP on cyclic GMP levels

Incubation of neutrophils with the NO-releasing compounds, GEA 3162 and GEA 5024 (1–100 μM), as well as fMLP (0.1–0.3 μM), for 10 min at 37°C induced concentration-dependent and significant increases in cyclic GMP production ($P<0.05$). Both GEA 3162 and GEA 5024 were found to be more potent than fMLP at increasing cyclic GMP production in human neutrophils (Table 1). SIN-1 was considerably less potent than the GEA compounds (data not shown).

Effect of the NO-releasing compounds and fMLP on nitrate/nitrite

Incubation of neutrophils with the NO-releasing compounds, GEA 3162, GEA 5024 and SIN-1 (1–100 μM) for 30 min at

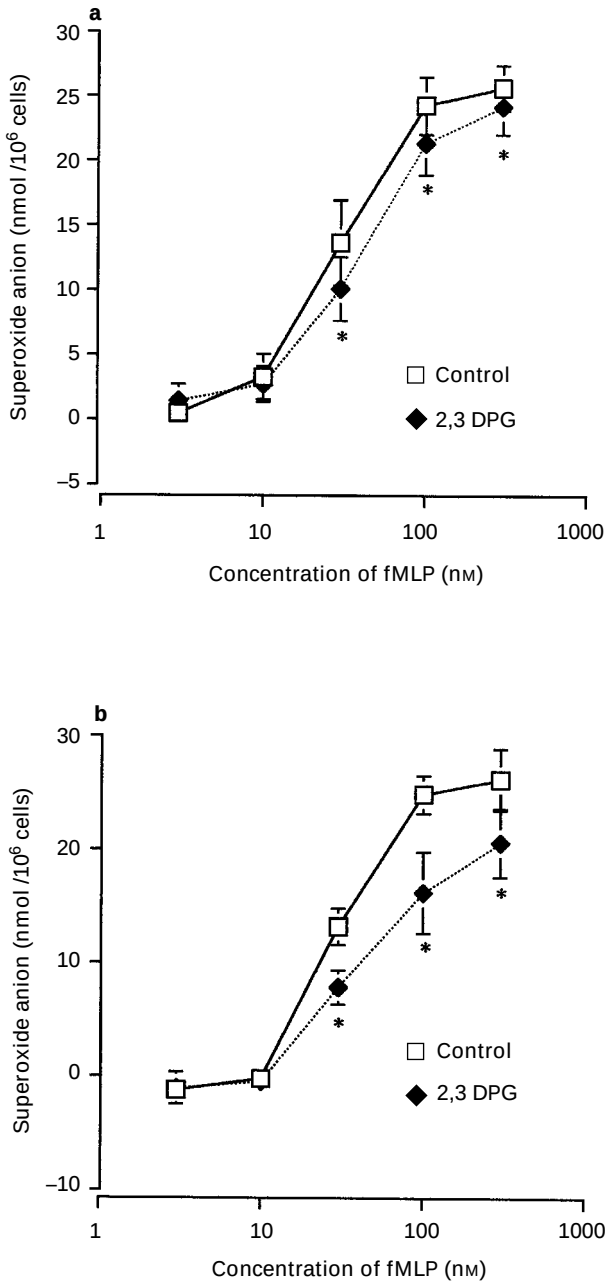

Figure 8 Log concentration-effect curves for fMLP induced superoxide anion generation in control cells and cells treated with DPG (a) 100 μM and (b) 500 μM. The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $*P<0.05$.

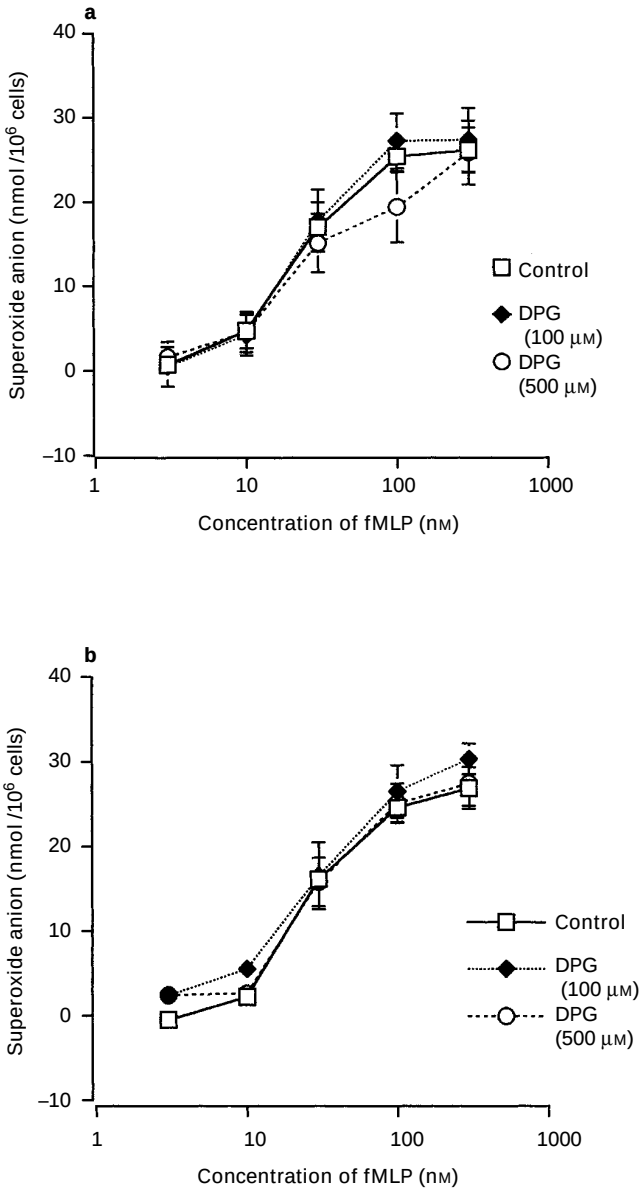

Figure 9 Log concentration-effect curves for fMLP induced superoxide anion generation in control cells and cells treated with 2,3-diphosphoglyceric acid (DPG) 100 μM and 500 μM. Unlike Figure 8, here cells were preincubated at 37°C with DPG for (a) 10 min and (b) 20 min. The values are the mean, and vertical lines show s.e.mean, of 4 different donors.

37°C induced concentration-related increases in total nitrate/nitrite production (Table 2). GEA 3162 and GEA 5024 were less potent than SIN-1. With GEA 3162 as the standard agonist ($EC_{50}=39.70\pm0.53$ μM), apparent EC_{50} values calculated for SIN-1 and GEA 5024 were 37.62 ± 0.9 ($n=4$; EEC of 0.95) and 89.86 ± 1.62 μM ($n=4$; EEC of 2.26), respectively. fMLP at concentrations of 3–300 nM caused no significant increase in total nitrate/nitrite.

Discussion

In the neutrophil, several second messenger/signal transduction systems can become activated, and these may be involved in the regulation of a variety of neutrophil effector functions. It has been shown that fMLP-induced chemotaxis in human neutrophils results from a rise in cyclic GMP levels subsequent to the production of NO (Kaplan *et al*., 1989; Belenky *et al*., 1993). Such a role for NO has been supported by our results here where inhibition of NOS with L-NMMA (Figure 1a) and chemical antagonism of NO with the NO scavenger carboxy-PTIO (Akaike *et al*., 1993) (Figure 1b) inhibited fMLP-induced chemotaxis. However, it must be noted that high concentrations of both of these agents were used (500 and 100 μM, respectively), suggesting that NO represents only one of the pathways by which chemotaxis is induced, as complete block of NO only partially blocked the chemotactic response of fMLP.

It has been shown that cyclic GMP and G-kinase regulate neutrophil activation in response to fMLP or A-23187 (Pryzwansky *et al*., 1990; Wyatt *et al*., 1990). After activation by fMLP, G-kinase transiently co-localizes with the intermediate filaments, resulting in the phosphorylation of its substrate protein, vimentin (Wyatt *et al*., 1991). LY 83583 is an inhibitor of guanylyl cyclase and has been shown to inhibit the fMLP-stimulated increase in neutrophil cyclic GMP levels resulting in inhibition of the co-localization and subsequent phosphoryla-

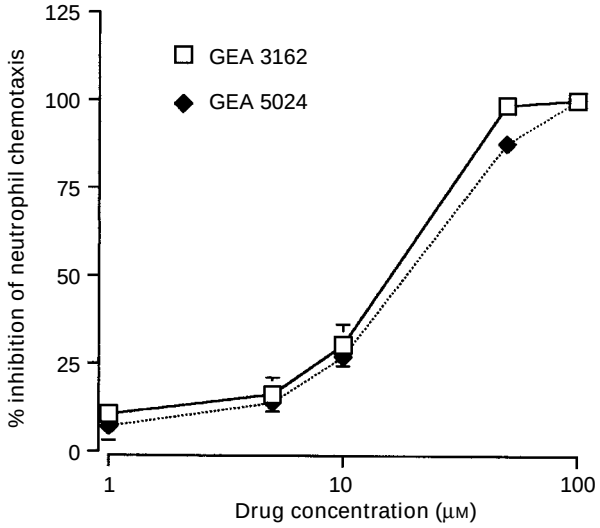

Figure 10 Log concentration-effect curves for inhibition of fMLP-induced chemotaxis, observed with GEA 3162 and GEA 5024. Cells were preincubated with GEA 3162 and GEA 5024 for 10 min at 37°C before being added to the chemotaxis chamber. The values are the mean, and vertical lines show s.e.mean, of 5 different donors.

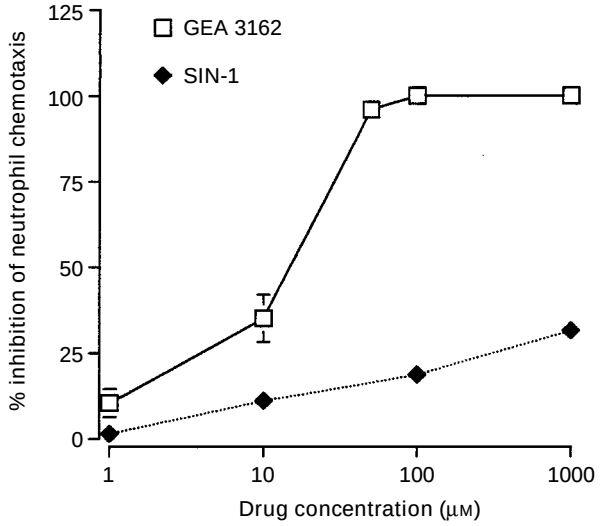

Figure 11 Log concentration-effect curves for inhibition of fMLP-induced chemotaxis, observed with GEA 3162 and SIN-1. Cells were preincubated with GEA 3162 and SIN-1 for 10 min at 37°C before being added to the chemotaxis chamber. The values are the mean, and vertical lines show s.e.mean, of 8 different donors.

Table 1 The effects of NO releasing compounds (GEA 3162 and GEA 5024) and fMLP on cyclic GMP levels in human neutrophils

Drug concentration (μM)	*Cyclic GMP levels* (pmol/10^6 cells) *GEA 3162*	*GEA 5024*	*fMLP*
0	0.1±0.02	0.1±0.01	0.09±0.01
0.1			0.18±0.03*
0.3			0.22±0.05*
10	0.87±0.15***	0.59±0.11**	
50	1.13±0.23***	0.73±0.14**	
100	1.32±0.24***	0.85±0.18**	

Data are expressed as mean±s.e.mean of 5 different donors. Significant increase in cyclic GMP above basal, $*P<0.05$, $**P<0.01$, $***P<0.005$.

Table 2 The effects of NO releasing compounds (GEA 3162, GEA 5024 and SIN-1) on total nitrate/nitrite production in human neutrophils

Drug concentration (μM)	*Total nitrate/nitrite* (μmol/10^6 cells) *GEA 3162*	*GEA 5024*	*SIN-1*
0	5.8±3.3	5.8±3.3	5.8±3.3
1	11.3±4.8	2.8±0.0*	9.4±4.6**
10	71.8±3.6	30.5±0.6**	55.4±1.6**
50	282.2±5.5	145.0±1.4**	$305.2\pm5.1^{\#}$
100	458.0±3.6	251.4±1.6**	$552.0\pm14.7^{\#\#}$

Data are expressed as mean±s.e.mean of 4 different donors. *Significantly less nitrate/nitrite production than the equivalent concentration of GEA 3162, $*P<0.05$, $**P<0.005$. $^{\#}$Significantly more nitrate/nitrite production than the equivalent concentration of GEA 3162, $^{\#}P<0.01$, $^{\#\#}P<0.005$. GEA 3162 significantly increased nitrate/nitrite levels at all concentrations tested.

tion of vimentin by G-kinase (Wyatt *et al*., 1993). We found that LY 83583 significantly attenuated fMLP-induced chemotaxis (Figure 2a and b) confirming that the co-localization and phosphorylation of G-kinase and vimentin are involved in fMLP-induced neutrophil chemotaxis. Two inhibitors of G-kinase were used to substantiate such a role for cyclic GMP, KT 5823 (Kase *et al*., 1987) and Rp-8-pCPT-cGMPS (Butt *et al*., 1994). At a concentration of 10 μM (Figure 3a) KT 5823 completely inhibited fMLP-induced chemotaxis. The block observed with both LY 83583 (100 μM) and KT 5823 (10 μM) was greater than would be expected if the rise in cyclic GMP resulted only from NO. This suggests either that fMLP can increase cyclic GMP levels independently of NO or that these agents are not acting as selective inhibitors of guanylyl cyclase and G-kinase at the concentrations used. Rp-8-pCTP-cGMPS (10 and 100 μM) failed to block fMLP-induced chemotaxis, suggesting that the G-kinase activated in neutrophil chemotaxis is not type Glα. The inability of Rp-8-pCPT-cGMPS to block chemotaxis is unlikely to result from too low a concentration being used as a significant effect was observed with Rp-8-pCPT-cGMPS (100 μM) on SAG (Figure 7).

It has been shown that more than 90% of the diglyceride formed in neutrophils in response to fMLP occurs through the activation of phospholipase D (PLD)/phosphatidic acid (PA) phosphohydrolase (Billah *et al*., 1989). A phosphatase inhibitor, DPG, significantly attenuated fMLP-induced chemotaxis (Figure 4a and b), suggesting that activation of PLD is a major signal in neutrophil chemotaxis. It is not clear from these results whether increased intracellular Ca^{2+} resulting from PLD activation is the trigger responsible for activation of NOS.

However the roles of NO and cyclic GMP are less clear in fMLP-induced SAG. Two NOS inhibitors were investigated and neither L-NMMA nor L-canavanine inhibited fMLP-induced SAG, even when used at the concentration (500 μM) required to inhibit neutrophil chemotaxis. However, the NO scavenger carboxy-PTIO (100 μM) significantly inhibited fMLP-induced SAG (Figure 5). The reason for this discrepancy is not clear, but these results suggest that NO may also play a role in SAG by fMLP.

Results with the guanylyl cyclase inhibitor LY 83583 suffer from the ability of LY 83583 to enhance significantly the amount of SAG by low concentrations of fMLP (3–10 nM), consistent with data showing that LY 83583 can itself stimulate SAG. However, both inhibitors of G-kinase, KT 5823 (Figure 6a and b) and Rp-8-pCPT-cGMPS (Figure 7) significantly inhibited fMLP-induced SAG. As with chemotaxis, the effect of KT 5823 was quite dramatic, suggesting that cyclic GMP may play an additional role to NO. However, the selectivity of the inhibitors used is crucial to this interpretation and experiments looking at phosphorylation of G-kinase are required to substantiate these findings. Furthermore, the dif-

ferent sensitivity to these G-kinase inhibitors observed with chemotaxis and SAG suggests that the G-kinase activated in the two processes may be different. Consistent with this, KT 5823 has been found not to inhibit the neutrophil G-kinase which phosphorylates vimentin (Wyatt & Pryzwensky, 1991).

PLD is thought to play a major signalling role in SAG in the primed neutrophil (Bonser *et al*., 1989; Kanaho *et al*., 1993). In particular PLD is thought to ensure that diacylglycerol levels are sustained, which is a requirement for SAG (Billah & Anthes, 1990). Such a role for PLD has been confirmed in these experiments by use of the phosphatase inhibitor DPG, which significantly inhibited fMLP-induced SAG in these cytochalasin B-treated neutrophils (Figure 8a and b). This effect of DPG was lost if cells were pre-incubated with DPG for 10 or 20 min (Figure 9a and b). This result contrasts with those obtained by Kanaho *et al*. (1993) who observed greater block with preincubation. A greater degree of block was observed with chemotaxis than with SAG.

While these results suggest that endogenous NO plays a role in mediating neutrophil chemotaxis, other evidence has been presented indicating that NO releasing compounds can inhibit neutrophil activation (Ney *et al*., 1990; Schroder *et al*., 1990; Kubes *et al*., 1991; Wenzen-Seifert *et al*., 1991). To resolve this apparent paradox we have investigated the effects of GEA 3162, GEA 5024 and SIN-1 which have previously been shown to inhibit neutrophil chemotaxis (Moilanen *et al*., 1993). GEA 3162 and GEA 5024 (1–100 μM) caused significant concentration-dependent inhibition of fMLP-induced chemotaxis (Figure 10). SIN-1 was less potent and caused significantly less inhibition of chemotaxis than GEA 3162 (Figure 11). The rank order of potency was GEA 3162 ($EC_{50} = 14.7 \pm 1.58$ μM) > GEA 5024 ($EC_{50} = 18.4 \pm 0.43$ μM) > SIN-1 ($EC_{50} = >$ 1000 μM). One possible explanation for the difference in potency of these agents as inhibitors of chemotaxis may relate to the ability of SIN-1 to release superoxide anion (Feelisch *et al*., 1989; Feelisch, 1991). The concomitant release of NO and superoxide anion by SIN-1 may well attenuate the inhibitory effects of NO on chemotaxis as NO is inactivated by superoxide anions, to form peroxynitrite (Gryglewski *et al*., 1986). Furthermore, peroxynitrite production by SIN-1 has been shown to enhance fMLP-induced neutrophil respiratory burst (measured as luminol-dependent chemiluminescence in whole blood) masking its otherwise inhibitory effects, such as a reduction in leukotriene B_4 production (Bednar *et al*., 1996). In addition peroxynitrite formed from SIN-1 has been shown to stimulate phorbol ester-induced respiratory burst (Iha *et al*., 1996). In contrast, GEA 3162 and GEA 5024 (in concentrations up to 100 mM) do not release significant amounts of superoxide anions to form peroxynitrite. If peroxynitrite augments chemotaxis as well as SAG, this could explain why GEA 3162 and GEA 5024 are more potent inhibitors of neutrophil chemotaxis than SIN-1.

The NO releasing compounds increased total nitrate/nitrite production (Table 2) with a rank order of potency of SIN-1 ($EC_{50} = 37.62 \pm 0.9$ μM) > GEA 3162 ($EC_{50} = 39.7 \pm 0.5$ μM) > GEA 5024 ($EC_{50} = 89.9 \pm 1.7$ μM). Taking GEA 3162 as the standard compound, this gives EEC values for SIN-1 and GEA 5024 of 0.95 and 2.26 for nitrate/nitrite production compared to >62.7 and 1.25 for inhibition of chemotaxis.

Clearly some reason is required to explain the lack of potency of SIN-1 at inhibiting chemotaxis compared with releasing NO. Interestingly, as found by Moilanen *et al*. (1993), SIN-1 was much weaker than GEA 3162 and GEA 5024 at increasing cyclic GMP levels, giving a maximal increase of 1.4 fold over basal (data not shown) compared with 13.2 and 8.3 fold for GEA 3162 and GEA 5024, respectively (Table 1). Consequently, there is a better correlation between effects on cyclic GMP and inhibition of chemotaxis, than for effects on NO and inhibition of chemotaxis. At first glance, this suggests that neutrophil inhibition is likely to be related to increased cyclic GMP levels rather than ADP ribosylation by NO (Clancy *et al*., 1995), but the role of peroxynitrite formed by SIN-1 requires further clarification, particularly with respect to chemotaxis. At present it is not clear whether peroxynitrite augments chemotaxis induced by fMLP as is the case for SAG (Iha *et al*., 1996).

These results do not prove that GEA 3162, GEA 5024 and SIN-1 inhibit neutrophil chemotaxis by a NO-dependent mechanism. However, this is quite difficult to test. A NO scavenger such as carboxy-PTIO will itself inhibit chemotaxis (Figure 1b), so that the NO donor would be tested against a smaller fMLP stimulus. Because of the nature of physiological antagonism, it is easier to inhibit a smaller stimulus than a larger one (Kenakin, 1987) making comparison difficult. The scavenger oxyhaemoglobin may prove useful in elucidating the role of NO, if this can be used at a low enough concentration not to affect endogenous NO and the control chemotactic response to fMLP.

In conclusion, these results confirm that neutrophil activation results from the stimulation of several signal transduction systems. We have shown that chemotaxis can be attenuated by inhibitors of PLD, NO and cyclic GMP. It appears that increases in cyclic GMP and activation of G-kinase resulting in chemotaxis can occur via a NO-dependent as well as NO-independent pathway. As such, small increases in cyclic GMP but not NO were detectable after neutrophil stimulation by fMLP. Similar pathways appear to operate in SAG. In contrast, the NO releasing compounds, GEA 3162, GEA 5024 and SIN-1, which produce large amounts of NO (measured as total nitrate/nitrite) compared to fMLP, inhibit neutrophil chemotaxis. This hypothesis, that low concentrations of NO activate while high concentrations inhibit neutrophils, has also been suggested recently by VanUfflen *et al*. (1996); they studied the effects of gaseous NO on rabbit peritoneal neutrophils.

P.W. is funded by a Royal Thai Government Scholarship. This work is supported by Allergan Inc.

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(Received June 26, 1997
Revised August 4, 1997
Accepted August 8, 1997)