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Reduction by inhibitors of mono(ADP-ribosyl)transferase of chemotaxis in human neutrophil leucocytes by inhibition of the assembly of filamentous actin

Jennifer R. Allport, Louise E. Donnelly, *Brian P. Hayes, Stephen Murray, Nigel B. Rendell, *Keith P. Ray & [1]John MacDermot

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Du Cane Road, London, W12 0NN and *Cell Biology, Biology Division, Glaxo-Wellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts, SG1 2NY

1 Chemotaxis of human neutrophils is mediated by numerous agents [e.g. N-formyl-methionyl-leucyl-phenylalanine (FMLP) and platelet activating factor (PAF)] whose receptors are coupled to phospholipase C. However, the subsequent transduction pathway mediating cell movement remains obscure. We now propose involvement of mono(ADP-ribosyl)transferase activity in receptor-dependent chemotaxis.

2 Human neutrophils were isolated from whole blood and measurements were made of FMLP or PAF-dependent actin polymerization and chemotaxis. The activity of cell surface Arg-specific mono(ADP-ribosyl)transferase was also measured. Each of these activities was inhibited by vitamin K_3 and similar IC_{50} values obtained (4.67 ± 1.46 μM, 2.0 ± 0.1 μM and 4.7 ± 0.1 μM respectively).

3 There were similar close correlations between inhibition of (a) enzyme activity and (b) actin polymerization or chemotaxis by other known inhibitors of mono(ADP-ribosyl)transferase, namely vitamin K_1, novobiocin, nicotinamide and the efficient pseudosubstrate, diethylamino(benzylidineamino)guanidine (DEA-BAG).

4 Intracellular Ca^{2+} was measured by laser scanning confocal microscopy with two fluorescent dyes (Fluo-3 and Fura-Red). Exposure of human neutrophils to FMLP or PAF was followed by transient increases in intracellular Ca^{2+} concentration, but the inhibitors of mono(ADP-ribosyl)transferase listed above had no effect on the magnitude of the response.

5 A panel of selective inhibitors of protein kinase C, tyrosine kinase, protein kinases A and G or phosphatases 1 and 2A showed no consistent inhibition of FMLP-dependent polymerization of actin.

6 We conclude that eukaryotic Arg-specific mono(ADP-ribosyl)transferase activity may be implicated in the transduction pathway mediating chemotaxis of human neutrophils, with involvement in the assembly of actin-containing cytoskeletal microfilaments.

Keywords: Neutrophil leucocytes; mono(ADP-ribosyl)transferase; actin polymerization; chemotaxis; calcium

Introduction

The cellular events mediating chemotaxis are complex. There is continuous realignment of the microfilamentous elements of the cytoskeleton, contraction of actin-containing microfilaments and adhesion or release of defined regions of the cell surface to extracellular matrix (Stossel, 1993; Wilkinson & Haston, 1988). An early event in chemotaxis involves a shape change in which the migrating cell becomes elongated. The process is accompanied by polarization of the receptors for the chemoattractant to the leading edge of the cell (reviewed in Wilkinson & Haston, 1988).

The receptors that recognize chemoattractants and trigger cell movement of human polymorphonuclear neutrophil leucocytes (PMNs) are all coupled to inositol phosphate (IP)-specific phospholipase C (PLC) and include receptors for N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Boulay *et al.*, 1990), platelet activating factor (PAF) (Nakamura *et al.*, 1991), leukotriene B_4 (LTB_4) (Sherman *et al.*, 1992), complement fragment C5a (Gerard & Gerard, 1991) and interleukin-8 (IL-8) (Thomas *et al.*, 1991). Activation of each of these receptors mediates hydrolysis of phosphatidylinositol 4,5-*bis*phosphate to yield 1,2-diacylglycerol (DAG) and inositol-1,4,5-*tris*phosphate (IP_3). These two intracellular second messengers mediate activation of protein kinase C (PKC) and release of Ca^{2+} from endoplasmic reticulum, respectively (reviewed in Berridge, 1993).

The roles of $[Ca^{2+}]_i$ or PKC in the signalling pathway mediating polymerization of filamentous actin or chemotaxis remain unresolved and the published findings of their effects in PMN chemotaxis are ambiguous (Zimmerman *et al.*, 1988; Gaudry *et al.*, 1988). PKC has been implicated in the facilitation of chemotaxis or actin polymerization by phorbol ester or DAG (Wright *et al.*, 1988), and inhibition by selected PKC inhibitors (Gaudry *et al.*, 1988). Conversely, other reports describe (i) actin polymerization triggered by PKC inhibitors (Keller *et al.*, 1990), and (ii) no capacity of phorbol ester to mimic FMLP-dependent actin polymerization (Howard & Wang, 1987). Similar ambiguity relates to changes in $[Ca^{2+}]_i$ and actin polymerization. Numerous studies have implicated changes in $[Ca^{2+}]_i$ in receptor-mediated chemotaxis of PMNs (e.g. Bouchelouche *et al.*, 1990), although there are reports also of (a) chemotaxis mediated by FMLP with no change in $[Ca^{2+}]_i$ (Al-Mohanna *et al.*, 1990), and (b) disassembly of actin polymers mediated by rises in $[Ca^{2+}]_i$ (Bengtsson *et al.*, 1993). In summary, the cell surface receptors that recognize a chemotactic gradient are coupled to activation of PLC, although the subsequent involvement of increases in $[Ca^{2+}]_i$ or activation of PKC are unclear.

Many of the activities of PMNs are inhibited by vitamin K and its analogues. Vitamins K_3 and K_5 inhibit PMN spreading, chemotaxis, phagocytosis, degranulation and antibacterial activity. In addition, there is inhibition of the generation of superoxide and hydrogen peroxide (Gallin *et al.*, 1982; Shakarjian & Carchman, 1990). Paradoxically, vitamin K_3 has been reported to stimulate the hexose monophosphate shunt in

[1] Author for correspondence.

PMNs, and amplify the $[Ca^{2+}]_i$ transient mediated by chemoattractant (Gallin *et al.*, 1982). The effects of vitamin K analogues are mediated by oxidation or conjugation of critical sulphydryl groups by vitamin K epoxide, or reduction of the quinone moiety of vitamin K. The molecule provides a potent electron acceptor that interferes with electron transport (Gallin *et al.*, 1982). The molecular target of vitamin K in PMNs is unknown, but vitamin K is one of numerous inhibitors of mono(ADP-ribosyl)transferase (EC 2.4.2.31). The enzyme mediates a two-step reaction involving first hydrolysis of NAD^+ to yield nicotinamide and ADP-ribose. Thereafter an adduct is formed between ADP-ribose and a guanidino nitrogen of specific polypeptide arginine residues. The free nicotinamide will act as an inhibitor of further mono(ADP-ribosyl)transferase activity. The catalytic activity of Arg-specific mono(ADP-ribosyl)transferase is similar to that of cholera toxin, although the two enzymes are structurally unrelated (reviewed in Ueda & Hayaishi, 1985).

Eukaryotic mono(ADP-ribosyl)transferase has been cloned from human (Okasaki *et al.*, 1994) and rabbit skeletal muscle (Zolkiewska *et al.*, 1992), and unspecified cells derived from avian bone marrow (Tsuchiya *et al.*, 1994). They show significant structural homology to each other, and are now known to be membrane-bound proteins that are tethered to the lipid bilayer by a glycosylphosphatidylinositol (GPI) anchor (Okasaki *et al.*, 1994). GPI-linked proteins are confined to the outer aspect of the plasma membrane of eukaryotic cells (Ferguson, 1992), with the exception of ornithine decarboxylase (Mustelin *et al.*, 1987) and GP-2 (a major integral protein of pancreatic zymogen granules) (Ronzio *et al.*, 1978) which are located on the inner aspect of the plasma membrane and the lumenal surface of the granule respectively. The substrates of eukaryotic mono(ADP-ribosyl)transferases in intact cells are largely unknown, although in mouse skeletal myocytes the substrate has been identified as the laminin-binding protein, integrin α7 (Zolkiewska & Moss, 1993).

In the present paper, we describe experiments with human neutrophils which suggest a role for mono(ADP-ribosyl)-transferase activity in receptor-dependent chemotaxis and polymerization of monomeric (globular G) actin to form filamentous (F) actin of mature microfilaments.

Methods

The isolation of human PMNs

Blood was taken by venous puncture from male or female human volunteers (aged 21–48 years), and the protocol was approved by the local ethics committee. Subjects taking paracetamol, aspirin, ibuprofen or other non-steroidal anti-inflammatory drugs were excluded. PMNs were isolated by a modification of the method of Haslett *et al.* (1985). Blood was mixed with a 0.15× volume of 3.8% (w/v) sodium citrate, and the erythrocytes were allowed to sediment through a 4:1 (v/v) Hespan:citrate buffer (0.9% (w/v) saline containing 10 mM tri-sodium citrate, 0.1 mM sodium di-hydrogen phosphate and 1 mM glucose) solution for 45 min at room temperature. The leucocyte rich plasma (LRP) was removed, and centrifuged at 400 *g* for 5 min. The resulting pellet was resuspended in 2.5 ml plasma per 20 ml starting volume of whole blood. LRP (2.5 ml volumes) was separated by centrifugation at 500 *g* for 30 min on discontinuous Percoll gradients comprising 5 ml 81%, 3 ml 70% and 2.5 ml 55% (v/v) Percoll in phosphate buffered saline (PBS). The platelets are separated from the other cellular components of blood during centrifugation, and are located above the 55% (v/v) Percoll layer. The PMNs were removed from the interface between 70% and 81% (v/v) Percoll, washed in PBS and resuspended in Hanks' balanced salts solution (HBSS; Ca^{2+}/Mg^{2+}-free). Cells were left at room temperature for 1 h prior to use in experiments.

Measurement of chemotaxis

Chemotaxis of PMNs was measured in a 48 well micro-chemotaxis chamber by a modification of the methods of Wilkinson (1988) and Falk *et al.* (1980). PMNs were resuspended at 2×10^6 cells ml^{-1} in HBSS containing 0.1% (w/v) bovine serum albumin (BSA) and 30 mM HEPES buffer, pH 7.2 (suspension buffer). The chemotaxis chamber was pre-equilibrated to 37°C. The lower wells of the chamber were loaded with 25 μl of suspension buffer containing FMLP at the concentrations indicated. Cell suspension (50 μl) was loaded into the upper wells and the chamber was incubated at 37°C for 1 h to allow migration of the cells through the filter. The filter was then removed, and the cells in the filter fixed in 70% (v/v) ethanol. The PMN nuclei were stained with haematoxylin, and the filter dehydrated with sequentially increasing concentrations of ethanol. The filters were rendered transparent in xylene, and then mounted onto glass slides with DePex mounting medium. The same field was chosen at the centre of each well to ensure that counting was not biased, and all cells within that field that had migrated more than 20 μm into the filter were counted.

Measurement of actin polymerization

The abundance of polymerized actin was measured by a modification of the method of Howard & Meyer (1984), which involves the binding of 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin to filamentous actin. LRP was isolated and cells resuspended at 5×10^6 cells ml^{-1} in HBSS containing 0.05% (w/v) BSA, and 25 mM HEPES buffer, pH 7.4. The cell suspension (80 μl) was preincubated at 37°C for 10 min to allow the cells to equilibrate. Cells were then incubated in the absence or presence of chemoattractant at 37°C for the times shown, and the reactions stopped by the addition of 100 μl 3.7% (w/v)

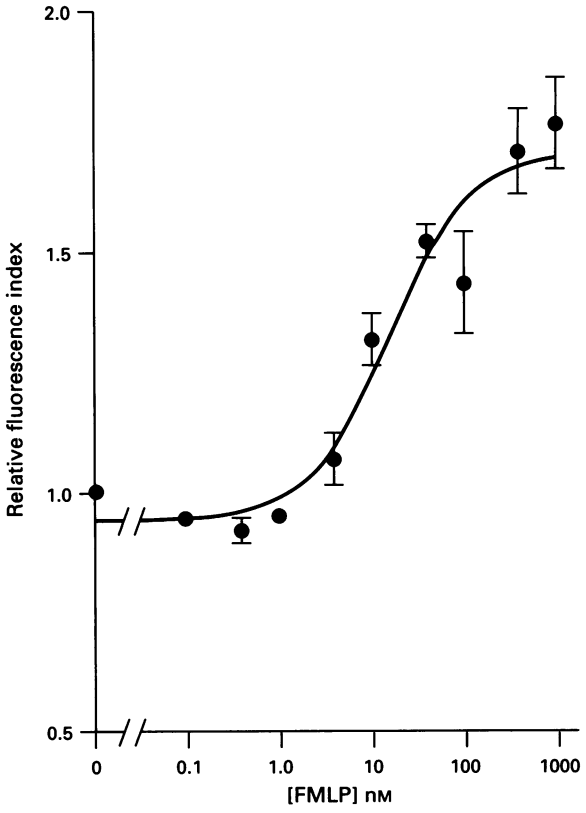

Figure 1 PMNs were prepared as described, and exposed to selected concentrations of FMLP or vehicle control for 1 min. Measurements were made of actin polymerization, and each value represents the means ± s.e.mean of triplicate determinations. The results shown are representative of 3 similar experiments with cells from separate donors.

paraformaldehyde in PBS containaing 0.2 mg ml^{-1} lysophosphatidylcholine and 0.3 μM NBD-phallacidin. The suspension was centrifuged at 250 g for 5 min at 4°C and the supernatant removed. The resulting pellet was washed twice with 500 μl PBS, and finally resuspended in 250 μl of PBS. The fluorescence of the NBD-phallacidin was quantified in an Epics Profile fluorescence activated cell sorter (FACS, Coulter Electronics), with an argon laser. The excitation wavelength (λ) was 488 nm and emission was recorded at 525 nm. Non-PMN cells, cell aggregates and cell debris were gated out. Approximately 3000 cells per sample were measured for their fluorescence, and the results expressed as the relative fluorescence index (RFI), which is defined as the fluorescence intensity of chemoattractant-activated cells compared to the fluorescence intensity of unstimulated cells. In preliminary experiments, we examined the polymerization of actin in isolated PMNs. Under these conditions, the fluorescent signal from NBD-phallacidin was very much greater in resting cells, and the signal which accompanied actin polymerization was correspondingly reduced. We therefore undertook further experiments with LRP, and gated the signal derived from PMNs using the FACS. We confirmed also that the addition of lymphocytes and monocytes to purified PMNs did not alter the fluorescent signal derived from PMNs.

Measurement of arginine specific mono(ADP-ribosyl)transferase activity – High performance liquid chromatography (h.p.l.c.) of [α^{32}P]-NAD$^+$

Initial measurements of mono(ADP-ribosyl)transferase activity revealed the necessity to re-purify commercially available [α^{32}P]-NAD$^+$. The method used was a modification of Formato *et al.* (1990). Chromatography was performed on a Nucleosil 10 C-18 column eluting with a linear gradient from 0–10% (v/v) methanol in 50 mM ammonium acetate buffer, pH 6.0. The flow rate was set at 1 ml min^{-1}, and fractions of 1 ml were collected. The peak corresponding to [α^{32}P]-NAD$^+$ (retention time ~19 min) was pooled, freeze-dried and resuspended in Dulbecco's modified Eagle's minimum essential medium (DMEM) as required.

Preparation of [^{3}H]-ADP-ribosylagmatine (internal standard)

Volumes of 500 μl containing 50 mM [^{3}H]-NAD$^+$ (25 μCi), 50 mM agmatine and 20 μg cholera toxin A (previously activated with 1 mM dithiothrietol) in 300 mM potassium phosphate buffer pH 7.0 were incubated at 37°C for 6 h. The [^{3}H]-ADP-ribosylagmatine product was purified by reverse phase h.p.l.c. Chromatography was performed on a Nucleosil 10 C-18 column with a linear gradient from 0.1% (v/v) trifluoroacetic acid to 30% (v/v) methanol in water. The sample was eluted at 1 ml min^{-1} and fractions of 1 ml were collected. The product (retention time ~ 11 min) was identified by u.v. absorbance at 254 nm and liquid scintillation counting. The fractions containing the [^{3}H]-ADP-ribosylagmatine were pooled and freeze dried. The product was resuspended in 20 mM Tris/HCl, pH 7.0 and stored at −20°C. The identity of the product was confirmed by electrospray mass spectrometry, an [M + H$^+$] ion was detected at m/z 674, corresponding to [^{3}H]-ADP-ribosylagmatine.

Measurement of mono(ADP-ribosyl)transferase activity

The method was taken from Moss & Stanley (1981) with modifications by McMahon *et al.* (1993). Isolated PMNs (5×10^6 cells) were resuspended in DMEM (without phenol red) containing 20 mM HEPES buffer, pH 7.0, 750 μM [α^{32}P]-NAD$^+$ (20 μCi per tube) and 50 mM agmatine in a final volume of 300 μl. Triplicate reactions were incubated at 37°C for the times indicated, and terminated by the addition of 300 μl of 2 M nicotinamide in 20 mM Tris/HCl buffer, pH 7.0 containing an internal standard of 5000 c.p.m. [^{3}H]-ADP-ribosylagmatine. The samples were centrifuged at 15,000 g for 30 min, and 500 μl of the supernatant was loaded on to 3 ml columns of

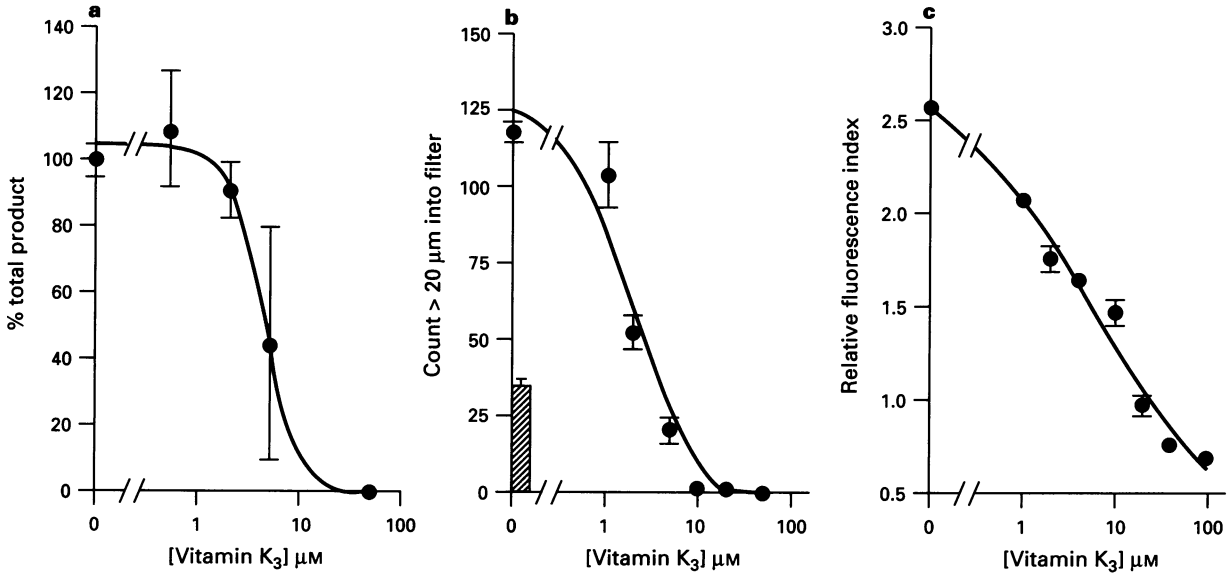

Figure 2 PMNs were prepared as described and allowed to rest for 1 h prior to experimentation. (a) PMNs (5×10^6) were incubated for 4 h at 37°C with 750 μM [α^{32}P]-NAD$^+$ and 50 mM agmatine in the presence of concentrations of vitamin K_3(1–100 μM) or vehicle control. The reactions were stopped with 2 M nicotinamide and the [^{32}P]-ADP-ribosylagmatine was separated from reagents by ion exchange chromatography. Each point is expressed as the mean ± s.e.mean (n=3). (b) PMNs were treated for 2 h with selected concentrations of vitamin K_3(1–100 μM) (●) or vehicle control (hatched column) and then allowed to migrate through cellulose nitrate filters (3 μm pore size) towards 100 nM FMLP. A single field was selected in each case, and the total number of cells that had migrated >20 μm were counted. Each point is expressed as the mean ± s.e.mean (n=4). (c) LRP was isolated as described and treated for 2 h with concentrations of vitamin K_3(1–100 μM) or vehicle control and then incubated at 37°C with 1 μM FMLP for 2 min. The cells were fixed as described in the methods and the polymerized actin in PMNs only was quantified. Each point is expressed as the mean ± s.e.mean (n=3). The results presented in (a), (b) and (c) are representative of 3 similar experiments with cells from separate donors.

Dowex 1 x 2-400 ion exchange resin (equilibrated with 20 mM Tris/HCl buffer, pH 7.0). The columns were then washed with 1 ml of 20 mM Tris/HCl buffer, pH 7.0, and the ADP-ribosylagmatine eluted with an additional 4 ml of 20 mM Tris/HCl buffer, pH 7.0. The amount of product (^{32}P counts) was corrected for losses on the column by quantification of the recovery of the internal standard, and enzyme activity was expressed as pmol [^{32}P]-ADP-ribosylagmatine per 10^6 cells h^{-1}.

Measurement of $[Ca^{2+}]_i$

In preliminary experiments measurement of $[Ca^{2+}]_i$ in human PMNs was performed with Fura 2 or Indo 1. Good signals were obtained, but these calcium sensitive dyes were found to be unsuitable for the proposed experiments. The maximum absorption or emission spectra of diethylamino(benzylidineamino)guanide (DEA-BAG, Soman *et al.*, 1986), vitamin K_3 and novobiocin were located within the range of excitation or emission of the two dyes. An alternative protocol was therefore adopted which involved measurement of $[Ca^{2+}]_i$ with Fluo-3 and Fura-Red. There was no interference pattern with DEA-BAG, vitamin K_3 or novobiocin.

In the revised protocol, increases in $[Ca^{2+}]_i$ were measured by a modification of the method of Lipp & Niggli (1993). This is also a ratiometric method for measurement of $[Ca^{2+}]_i$ by laser scanning confocal microscopy with two different fluorescent dyes. Fluo 3 is excited at 510 nm and emits at 540±15 nm, Fura Red is excited at 490 nm, and emits at >600 nm. The fluorescence of Fluo 3 increases, and the fluorescence of Fura Red decreases with binding of Ca^{2+}, generating a ratio of the two fluorescent signals which can be used to calculate the concentration of Ca^{2+} in the cytosol (Lipp & Niggli, 1993). PMNs were suspended at 5×10^6 cells ml^{-1} in HBSS buffer and loaded at 37°C for 30 min with 2 μM Fluo 3 and 10 μM Fura Red. The cells were washed in PBS, resuspended at 10^6 cells ml^{-1} in HBSS buffer containing 1 mM $CaCl_2$ and allowed to settle on to coverslips. Fluo 3 and Fura Red were excited at 490 nm and the emission recorded at 530 nm and 660 nm respectively. The cells were stimulated with either 10 nM FMLP or 100 nM PAF and the fluorescence monitored with the MagiCal system and TARDIS software (Applied Imaging International Ltd., Hylton Park, Sunderland, Tyne and Wear, SR5 3HD).

Statistical analyses

The correlations shown in Figure 4 were drawn using Fig P 6.0 (Fig P. Software Corp. U.S.A.). Graphs and calculations of IC_{50} and K_{act} (the substrate concentration required for 50% activation of the enzyme) were performed using GraphPAD InPlot 3.01 (GraphPAD Software, CA, U.S.A.). One way analysis of variance (Scheffe range test) was performed using StatGraphics (StatGraphics Corp. U.S.A.). Results throughout show mean values±s.e.mean.

Materials

DEA-BAG was synthesized according to the method of Soman *et al.* (1986). The purity of the reaction product was checked by t.l.c., using silica gel $60F_{254}$ as stationary phase and methanol as solvent. The identity of DEA-BAG was confirmed by desorption electron impact ionization mass spectrometry. Other materials were obtained from the following suppliers: Molecular Probes Inc., Eugene, OR, U.S.A. (fluorescent probes); Calbiochem-Novabiochem, Nottingham (PAF, staurosporine, genistein, tyrphostins A1 and A25, calphostin C, KT5720, KT5823, KT5926, okadaic acid); Roche Products Ltd, Welwyn Garden City (Ro 31-8220); New England Nuclear, Stevenage, Herts ([$\alpha^{32}P$]-NAD^+, [^3H]-NAD^+); Du Pont Pharmaceuticals, Letchworth, Herts (Hespan); Fisons, Loughborough, Leics (h.p.l.c. materials); GIBCO BRL, Paisley, Scotland (DMEM and salt solutions); Sartorius Instruments Ltd., Epsom,

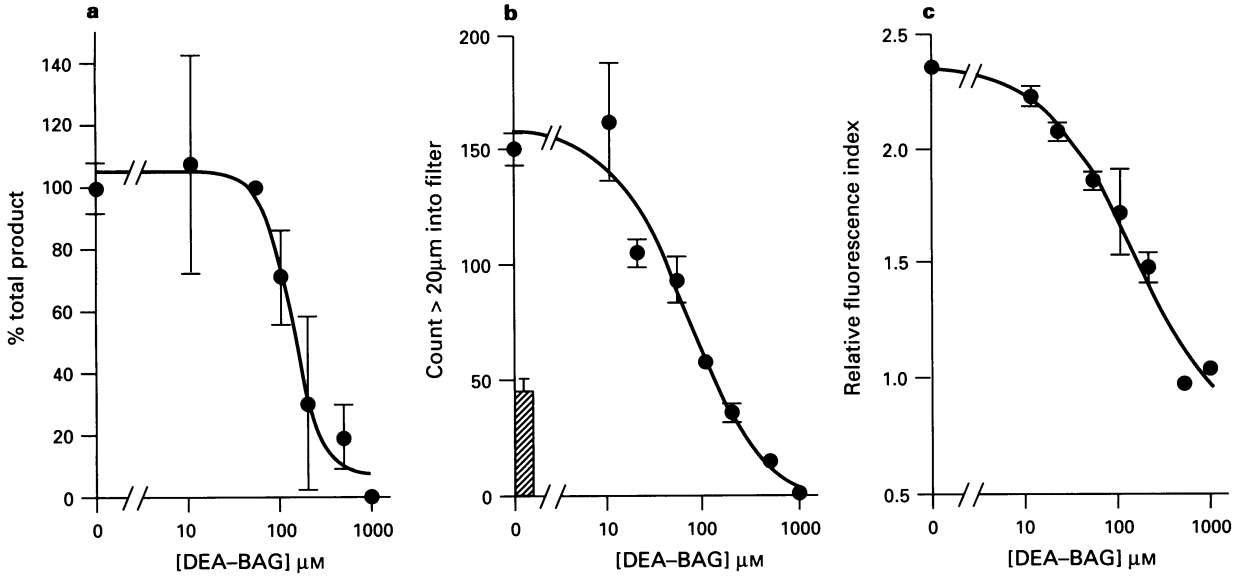

Figure 3 PMNs were prepared as described and allowed to rest for 1 h prior to experimentation. (a) PMNs (5×10^6) were incubated for 4 h at 37°C with 750 μM [$\alpha^{32}P$]-NAD^+ and 50 mM agmatine in the presence of concentrations of DEA-BAG (10–1000 μM) or vehicle control. The reactions were stopped with 2 M nicotinamide and the [^{32}P]-ADP-ribosylagmatine was separated from reagents by ion exchange chromatography. Each point is expressed as the mean±s.e.mean ($n=3$). (b) PMNs were treated for 2 h with selected concentrations of DEA-BAG (10–1000 μM) (●) or vehicle control (hatched column) and then allowed to migrate through cellulose nitrate filters (3 μm pore size) towards 100 nM FMLP. A single field was selected in each case, and the total number of cells that had migrated >20 μm were counted. Each point is expressed as the mean±s.e.mean ($n=4$). (c) LRP was isolated as described and treated for 2 h with concentrations of DEA-BAG (10–1000 μM) or vehicle control and then incubated at 37°C with 1 μM FMLP for 2 min. The cells were fixed as described in the methods and the polymerized actin in PMNs only was quantified. Each point is expressed as the mean±s.e.mean ($n=3$). The results presented in (a), (b) and (c) are representative of 3 similar experiments with cells from separate donors.

Surrey (cellulose nitrate filters for chemotaxis); Sigma Chemical Co., Poole, Dorset (vitamins K_1 and K_3, plus other biochemicals).

Results

Isolated human PMNs (>98% purity) were shown to be 97% viable by exclusion of trypan blue dye. The reagents employed in subsequent experiments had no effects on viability at the concentrations used. The time course of receptor-dependent actin polymerization revealed that the response reached a maximum 40 s after the addition of 1 μM FMLP. The maximum RFI values were revealed in a concentration curve for FMLP-dependent actin polymerization (Figure 1), and varied between 1.8 and 3.0. The concentration of FMLP mediating 50% of the maximum response (K_{act}) was 22 ± 9 nM ($n=3$). In subsequent experiments, saturating concentrations of FMLP (1 μM), PAF (1 μM) and C5a (4 nM) were used. Actin polymerization in the presence of 1 μM FMLP was inhibited by vitamin K_3, (Figure 2c), and the concentration of vitamin K_3 required for half maximum inhibition (IC_{50}) was 4.67 ± 1.46 μM ($n=3$). Inspection of Figure 2c shows that NBD-phallacidin fluorescence was reduced by vitamin K_3 below the level seen in the control cells (RFI < 1).

Measurements of PMN chemotaxis in response to FMLP showed a typical bell-shaped dose-response curve (data not shown). A similar response to FMLP was reported by Hoffstein *et al.* (1986). Maximum migration was observed at 100 nM FMLP, and the K_{act} was 1.14 ± 0.5 nM ($n=4$). Chemotaxis of PMNs in the presence of 100 nM FMLP was inhibited by vitamin K_3 (Figure 2b), with an IC_{50} of 2.0 ± 0.1 μM ($n=4$). High concentrations of vitamin K_3 (>10 μM) reduced cell migration below the levels seen in the absence of chemoattractant (i.e. both chemotaxis and chemokinesis were inhibited).

The ADP-ribosylation of agmatine by intact PMNs to yield mono(ADP-ribosyl)agmatine demonstrated the presence of Arg-specific mono(ADP-ribosyl)transferase activity. Enzyme activity was inhibited by vitamin K_3 (Figure 2a), with an IC_{50} of 4.7 ± 0.1 μM ($n=3$).

Further evidence supporting a role for mono(ADP-ribosyl)transferase in the control of actin polymerization and chemotaxis of PMNs was sought by experiments in which the cells were exposed to selected concentrations of DEA-BAG. DEA-BAG is an analogue of Arg that is an efficient pseudosubstrate of Arg-specific mono(ADP-ribosyl)transferase (Soman *et al.*, 1986). It has no α-amino group (unlike Arg or agmatine), and thus can not be ADP-ribosylated non-enzymatically by the addition of free ADP-ribose. Measurements were made of the capacity of DEA-BAG to inhibit mono(ADP-ribosyl)ation of agmatine, FMLP-dependent actin polymerization and FMLP-dependent chemotaxis (Figure 3). The protocol was similar to that shown in Figure 2. DEA-BAG inhibited ADP-ribosylation of agmatine with an IC_{50} value of 193 ± 40 μM ($n=3$; Figure 3a). DEA-BAG also inhibited actin polymerization ($IC_{50} = 213 \pm 2.0$ μM, $n=3$; Figure 3b) and chemotaxis ($IC_{50} = 147 \pm 78$ μM, $n=4$; Figure 3c) mediated by 1 μM FMLP.

A panel of known inhibitors of mono(ADP-ribosyl)-transferase activity were selected on the basis of their widely differing K_i values and molecular structures (Soman *et al.*, 1986; Rankin *et al.*, 1989; Banasik *et al.*, 1992). These included nicotinamide, novobiocin, vitamin K_1, vitamin K_3 and the pseudosubstrate, DEA-BAG. The effects of each compound were compared as inhibitors of (i) the mono(ADP-ribosyl)-ation of agmatine ($n=3$), (ii) chemotaxis mediated by 100 nM FMLP ($n=4$) and (iii) actin polymerization mediated by 1 μM FMLP, 1 μM PAF or 4 nM C5a ($n=3$). In each case, the IC_{50} values were determined from a full concentration curve of the selected inhibitors, and the results plotted as correlations between inhibition of enzyme activity and either chemotaxis (Figure 4a) or actin polymerization (Figure 4b). In each case, there was a close linear correlation between inhibition of mono(ADP-ribosyl)transferase activity and the measured biological responses ($r^2 = 0.82$ and 0.91 respectively; $P < 0.01$ in each case).

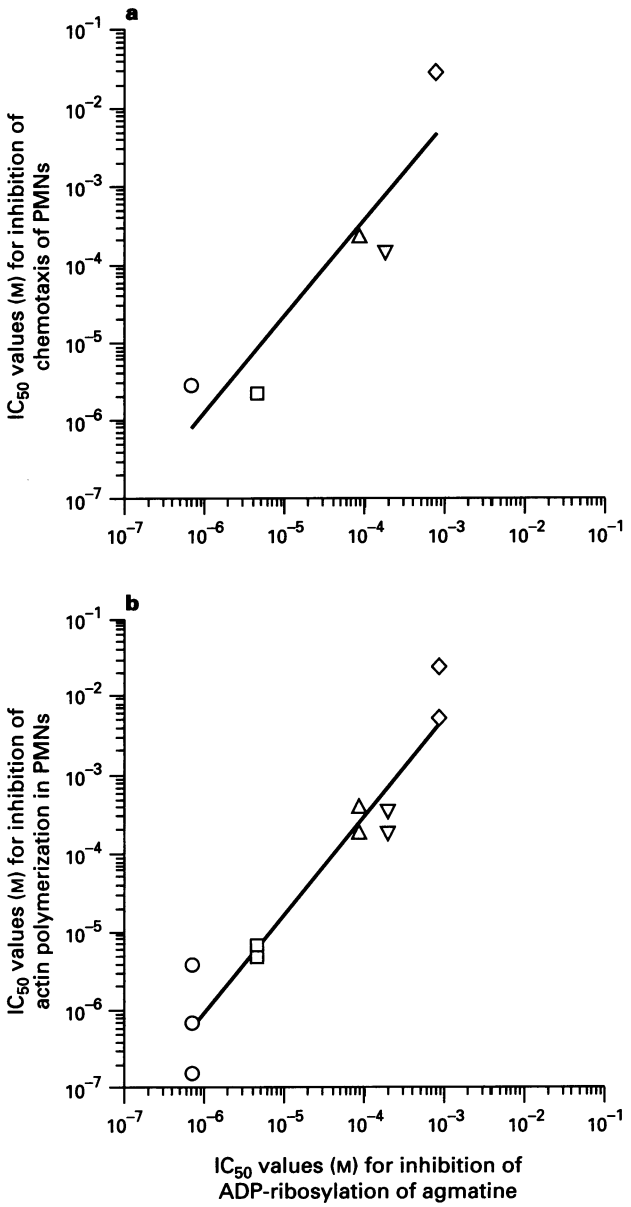

Figure 4 (a) Correlation between IC_{50} values measured for inhibition of PMN chemotaxis and IC_{50} values measured for inhibition of PMN mono(ADP-ribosyl)transferase. Each compound is defined by a symbol; nicotinamide (◇), novobiocin (△), vitamin K_1 (○), vitamin K_3 (□) and DEA-BAG (▽). Each point is the mean IC_{50} value for inhibition of PMN mono(ADP-ribosyl)transferase ($n=3$) against the IC_{50} values obtained for inhibition of FMLP-dependent chemotaxis ($n=4$). The linear correlation coefficient, $r^2 = 0.82$, $P < 0.01$. (b) Correlation between IC_{50} values measured for the inhibition of PMN mono(ADP-ribosyl)transferase and IC_{50} values measured for the inhibition of receptor-dependent actin polymerization in PMNs. Symbols as in (a). Each point is the mean IC_{50} value for inhibition of PMN mono(ADP-ribosyl)transferase against the mean IC_{50} values ($n=3$) obtained for inhibition of actin polymerization induced by each agonist (FMLP, PAF, C5a). The individual IC_{50} values were as follows: FMLP-dependent actin polymerization was inhibited by vitamin K_1 (3.82 ± 3.43 μM), vitamin K_3 (4.67 ± 1.46 μM), novobiocin (182 ± 2 μM), nicotinamide (5.3 ± 1.53 mM), DEA-BAG (213 ± 2 μM); PAF-dependent actin polymerization was inhibited by vitamin K_1 (0.67 ± 1.16 μM), novobiocin (367 ± 2 μM), nicotinamide (23.9 ± 1.7 mM); C5a-dependent actin polymerization was inhibited by vitamin K_1 (0.15 ± 1.79 μM), vitamin K_3 (6.57 ± 1.42 μM), DEA-BAG (129 ± 1 μM). The linear correlation coefficient, $r^2 = 0.91$, $P < 0.001$.

The possibility was considered that inhibitors of mono-(ADP-ribosyl)transferase activity might mediate their effects in these cells by alteration of the magnitude of the calcium transient following exposure of PMNs to chemoattractant. PMNs were exposed to 10 nM FMLP, which induced a rapid increase in $[Ca^{2+}]_i$ of 428±86 nM ($n=11$) that declined over 120 s. There was a similar response to 100 nM PAF with a maximum increase in $[Ca^{2+}]_i$ of 716±71 nM ($n=12$). Experiments were then performed to measure the FMLP or PAF-dependent increase in $[Ca^{2+}]_i$ in the absence or presence of vitamins K_1 (10 μM) or K_3 (20 μM), novobiocin (200 μM), nicotinamide (50 mM) or DEA-BAG (2 mM). The results shown in Table 1 indicate that there were no significant changes between any of the treatments.

The possibility was addressed that there might be other compounds that show similar tight correlations between inhibition of FMLP-dependent actin polymerization and inhibition of enzymes with more secure involvement in key pathways of signal transduction. Measurements were made of actin polymerization in PMNs following exposure to 1 μM FMLP, and the effects of selected concentrations of a panel of candidate inhibitors were examined (Table 2). Of the three PKC inhibitors examined, staurosporine and Ro 31-8220 had no effect, while calphostin C inhibited actin polymerization with an IC_{50} value of 113±56 nM ($n=3$). Three inhibitors of tyrosine kinase activity were examined, and the results show that only tyrphostin A1 inhibited actin polymerization with an IC_{50} value of 99±32 μM ($n=3$). Other inhibitors of tyrosine kinase, cyclic AMP-dependent protein kinase A, cyclic GMP-dependent protein kinase G, myosin light chain kinase and phosphatases 1 and 2A had no effects on actin polymerization.

Discussion

The signal transduction pathway mediating actin polymerization and realignment of the cytoskeleton during chemotaxis is largely unknown. Individual actin-containing microfilaments are assembled rapidly following exposure of PMNs to chemoattractant (Aderem, 1992; Stossel, 1993), and elongation of filament occurs by addition of monomeric G-actin elements to the blunt end of the growing microfilament adjacent to the leading edge of the cell (Bearer, 1993). The microfilaments are anchored to the plasma membrane by a complex assembly of proteins which are coupled to matrix-binding integrins spanning the lipid bilayer. The relevant matrix binding integrins adhere to extracellular fibronectin, collagen, etc. (Aderem, 1992; Stossel, 1993), and the anchoring point incorporates vinculin, talin α-actinin and other proteins (Aderem, 1992; Stossel, 1993). Migration during chemotaxis involves a process of adhesion of the leading edge of the cell to the substratum, contraction of actin-containing microfilaments and release of the trailing edge. The cell is thus drawn up through the gradient. Sequential extension of lamellipods in the direction of the gradient and realignment of the cytoskeleton are then required for continued cell migration.

Table 1 The increase in $[Ca^{2+}]_i$ above the basal level ($\Delta[Ca^{2+}]_i$) induced by 10 nM FMLP or 100 nM PAF following treatment of PMNs with mono(ADP-ribosyl)-transferase inhibitors and a pseudosubstrate (DEA-BAG)

Compound	$\Delta[Ca^{2+}]_i$ nM FMLP (10 nM)	PAF (100 nM)
None	401±69	741±74
Vitamin K_1 (10 μM)	377±47	720±10
Vitamin K_3 (20 μM)	275±130	1220±31
Novobiocin (200 μM)	–	705±89
Nicotinamide (50 mM)	720±46	926±31
DEA-BAG (2 mM)	909±51	815±9

The results are expressed as mean±s.e.mean; the number of replicate determinations varied between 2 and 14. There were no significant differences in the magnitude of the FMLP or PAF-dependent transients following treatment of the cells with the agents shown (one way analysis of variance-Scheffe range test).

Table 2 Effects of inhibitors of enzymes implicated in signal transduction on actin polymerization of PMNs in response to 1.0 μM FMLP

Compound	Target	IC_{50}
Staurosporine (0–1000 nM)	PKC (non-specific)	No effect
RO 31–8220 (0–10 μM)	PKC	No effect
Calphostin C (0–1000 nM)	PKC	113nM±56
Genistein (0–10 μM)	Tyrosine kinase (non-specific)	No effect
Tyrphostin A1 (0–100 μM)	Tyrosine kinase	99μM±32
Tyrphostin A25 (0–100 μM)	Tyrosine kinase	No effect
KT5823 (0–100 nM)	Cyclic GMP dep. PKG	No effect
KT5720 (0–1000 nM)	Cyclic AMP dep. PKA	No effect
KT5926 (0–1000 nM)	Myosin light chain kinase	No effect
Okadaic acid (0–1000 nM)	PP1+PP2A phosphatases	No effect

IC_{50} values are expressed as the mean±s.e.mean ($n=3$).

The results presented show that a panel of inhibitors of mono(ADP-ribosyl)transferase activity are also competent to inhibit receptor-dependent actin polymerization and chemotaxis of PMNs. Furthermore, there was a close linear correlation between inhibition of the enzyme on the surface of PMNs and inhibition of the biological responses. DEA-BAG is an efficient substrate for Arg-specific mono(ADP-ribosyl)transferase (Soman *et al.*, 1986), and the present results revealed similar IC_{50} values for inhibition of the ADP-ribosylation of agmatine and inhibition of actin polymerization and chemotaxis. These results provide compelling further evidence for a role for this enzyme in these responses. Several of the inhibitors tested (e.g. vitamin K_3, Figure 2) reduced the FMLP-dependent polymerization of actin and chemotaxis below the level seen in untreated cells. The simplest explanation for this observation is that the 'resting' cells were in fact activated above their ground state. The experimental protocol was modified to minimize this effect by allowing the cells to settle for 1 h at room temperature, followed by pre-incubation for 10 min at 37°C.

The substrate(s) of mono(ADP-ribosyl)transferase in human PMNs is unknown, but we considered the possibility that the substrate might be a G protein linked to the generation of the calcium transient (cf. the Cys-specific mono(ADP-ribosyl)transferase of *B. pertussis*). The FMLP and PAF-dependent rises in $[Ca^{2+}]_i$ were not however reduced by inhibitors of mono(ADP-ribosyl)transferase. The substrate of mono(ADP-ribosyl)transferase in mouse skeletal muscle is integrin α7 (Zolkiewska & Moss, 1993), which is a developmentally regulated protein that exists in tight assembly with integrin β1 to form a mature laminin-binding protein. We are currently exploring the possibility that other matrix binding integrins expressed on the surface of PMNs (or their related anchoring proteins) might be the substrates of mono(ADP-ribosyl)-transferase in these cells.

There is no evidence to support a process of activation of mono(ADP-ribosyl)transferase by cell surface receptors in any eukaryotic system. It is possible, however, that the enzyme

might be translocated to the cell surface following exposure of PMNs to chemoattractant. Alkaline phosphatase (another GPI-linked enzyme) is transferred to cell surface of PMNs following exposure to FMLP (Pezzi *et al.*, 1991). If mono-(ADP-ribosyl)transferase activity at the cell surface is similarly regulated, such a process would allow for the controlled ADP-ribosylation of specific substrates on the outer aspect of the plasma membrane.

Receptors for chemoattractants are coupled to PI-specific PLC, although the subsequent signalling events required for cell movement have yet to be established. We have highlighted the conflicting nature of published work in this field. The signal transduction pathway leading to the subsequent assembly of filamentous actin is largely unknown, although the initial step of receptor-dependent PLC activation suggests the possibility of downstream activation of PKC, tyrosine kinase, or changes in $[Ca^{2+}]_i$. The results presented here suggest no consistent effects of inhibitors of PKC or tyrosine kinase, since there was no correlation between the K_i values for inhibition of actin polymerization and inhibition of these enzymes. The idiosyncratic pattern of inhibition with calphostin C and tyrphostin A1 is suggestive only of the lack of specificity of these molecules for these enzymes. We considered the possibility of activation of several other enzymes implicated widely in signalling pathways, but further limited experiments suggested no involvement of PKA, PKG, myosin light chain kinase or phosphatases 1 or 2A.

The results presented here indicate that eukaryotic Arg-specific mono(ADP-ribosyl)transferase activity is involved in the transduction pathway mediating chemotaxis of PMNs, with involvement in the assembly of actin-containing cytoskeletal microfilaments.

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