Characterization of prostanoid receptors on rat neutrophils

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1 The effects of various prostanoid agonists have been compared on the increase in intracellular free calcium ($[Ca^{2+}]_i$) and the aggregation reaction of rat peritoneal neutrophils induced by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP).

2 Prostaglandin E_2 (PGE_2) and the specific IP-receptor agonist, cicaprost, both inhibited the FMLP-induced increase in $[Ca^{2+}]_i$ (IC_{50} 33 nM and 18 nM respectively) and the FMLP-induced aggregation reaction (IC_{50} 5.6 nM and 7.9 nM respectively). PGD_2 , $PGF_{2\alpha}$, and the TP-receptor agonist, U 46619, were inactive at the highest concentration tested (1 μ M).

3 The EP_1 -receptor agonist, 17-phenyl- ω -trilor PGE_2 , and the EP_3 -receptor agonists, GR 63799X and sulprostone, had no inhibitory effect on FMLP-stimulated rat neutrophils.

4 PGE_1 (EP/IP -receptor agonist) and iloprost (IP-receptor agonist) inhibited the FMLP-induced increase in $[Ca^{2+}]_i$ with IC_{50} values of 34 nM and 38 nM respectively. The EP_2 -receptor agonists, butaprost and misoprostol (1 μ M), inhibited both FMLP-stimulated $[Ca^{2+}]_i$ and aggregation. However another EP_2 -receptor agonist, AH 13205, was inactive in both assays.

5 Prostanoid receptors present on rat neutrophils were further characterized by measuring [3H]-adenosine 3':5'-cyclic monophosphate ([3H]-cyclic AMP) accumulation. Only those agonists capable of stimulating [3H]-cyclic AMP accumulation were able to inhibit both FMLP-stimulated $[Ca^{2+}]_i$ and aggregation.

6 These results indicate that rat neutrophils possess inhibitory IP and EP -receptors; the relative potencies of PGE_2 , misoprostol and butaprost are those expected for the EP_2 -receptor subtype. No evidence for DP, FP, TP or EP_1 and EP_3 -receptors was obtained.

Keywords: Prostaglandin E_2 ; prostacyclin; prostanoid receptors; cyclic AMP accumulation; intracellular free calcium; FMLP-stimulated aggregation; rat peritoneal neutrophils

Introduction

Neutrophils play a major role in the body's defence against infection and trauma and contribute towards pathophysiological conditions such as adult respiratory distress syndrome (ARDS) (Winder *et al.*, 1993), myocardial infarction and rheumatoid arthritis (Carlos & Harlan, 1990). Neutrophils can be recruited to areas of local inflammation in response to a variety of chemotactic stimuli such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), platelet-activating factor (PAF), complement fragment C5a, and leukotriene B_4 (LTB_4). Activation of neutrophils by chemotactic factors results in degranulation, NADPH oxidase-catalysed superoxide (O_2^-) production, and a variety of other cellular responses (De Togni *et al.*, 1984; Rossi, 1986).

Neutrophil responses can be modulated by prostaglandins (Gryglewski & Salvemini, 1992) with prostaglandins having both pro- and anti-inflammatory roles (Goodwin, 1991; Kitis *et al.*, 1991; Phipps *et al.*, 1991). Prostaglandins have been shown to have inhibitory effects on neutrophil responses *in vivo* (Fantone *et al.*, 1981) and *in vitro* (Fantone & Kinnes, 1983; Ham *et al.*, 1983; De Togni *et al.*, 1984; Kato *et al.*, 1986; Gryglewski *et al.*, 1987; Ney & Schrör, 1989, 1991; Nagata *et al.*, 1992). Prostaglandins inhibit only responses mediated by direct receptor activation and do not inhibit phorbol ester or A23187-induced responses (Fantone & Kinnes, 1983; De Togni *et al.*, 1984; Ney & Schrör, 1991).

Since adenosine 3':5'-cyclic monophosphate (cyclic AMP) also inhibits neutrophil function and is thought to provide negative feedback control of neutrophil activation (De Togni *et al.*, 1984; Verghese *et al.*, 1985; Nagata *et al.*, 1992; Nielsen *et al.*, 1992), it is presumed that the prostaglandin-

mediated effects may also be due to elevated neutrophil cyclic AMP resulting from activation of prostaglandin receptors. PGE_1 and PGE_2 have been shown to be consistently effective in inhibiting human neutrophil responses and recent evidence suggests that their effect is mediated via an EP_2 -receptor (Armstrong & Talpain, 1992; Wheeldon & Vardey, 1993) which is coupled positively to adenylyl cyclase (Coleman *et al.*, 1990). In contrast, it has been reported that stimulation of EP_3 -receptors (which are negatively coupled to adenylyl cyclase) by sulprostone leads to potentiation of opsonized zymosan-induced release of LTB_4 from human neutrophils (Wheeldon & Vardey, 1993) and directly stimulates chemotaxis (Armstrong, 1992).

The aim of the present study therefore was to characterize the major prostanoid receptors present on rat neutrophils, and to examine the EP -receptor in more detail. The effects of a comprehensive range of prostanoid receptor-selective agonists have been tested for potentiation or inhibition of neutrophil function, as measured by changes in FMLP-stimulated $[Ca^{2+}]_i$ and aggregation. These same agonists were also tested for effects on the production of cyclic AMP.

Methods

Preparation of neutrophils

Male, Sprague-Dawley rats fed *ad libitum* and weighing 190–230 g were used throughout this study. Peritoneal neutrophils were elicited by intraperitoneal injection of 3 ml of NIH thioglycollate broth (0.26 g ml⁻¹) 19 h prior to harvesting the cells (Fisker *et al.*, 1990). The rats were killed by cervical dislocation, and the leucocytes collected from the peritoneal cavity by lavage with 40 ml of ice-cold HEPES-

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buffered saline (HBS: composition, mM: NaCl 145, KCl 5, MgCl₂ 1, HEPES acid, 10, pH 7.55) supplemented with glucose (10 mM) and bovine serum albumin (1%, w/v). The cells were centrifuged at 20°C for 10 min at 300 g, and washed once in the same buffer. The cell suspension was then layered over Ficoll-paque and centrifuged at 20°C for 20 min at 700 g in a swing-out rotor. The resulting cell pellet contained $89 \pm 1\%$ neutrophils (assessed microscopically using Wright's stain). The main contaminating cell types were mononuclear leukocytes (Fisker *et al.*, 1990). The viability of cells in the final cell pellet was $94 \pm 1\%$ as judged by the Trypan blue exclusion test ($n = 19$).

Neutrophil aggregation

Neutrophil aggregation was measured with a Chrono-log platelet aggregometer set at 37°C and 800 r.p.m. (Ford-Hutchinson & Evans, 1988). The cells were resuspended in HBS supplemented with glucose (10 mM) to a cell density of 5×10^6 cells ml⁻¹. The aggregometer was adjusted to record 0% light transmission with unstimulated neutrophils and 100% transmission with 2.5×10^6 cells ml⁻¹. The cell suspension (500 µl) was incubated in siliconized cuvettes for 1 min with the addition of CaCl₂ (1 mM). Test drugs (5 µl) were then added for 2 min followed by FMLP (16 nM). Maximal aggregation was defined as the maximal change in light transmission during the 1–2 min following addition of FMLP and is expressed in arbitrary units (mm deflection of pen recorder). Control responses to FMLP alone were tested regularly throughout the course of each experiment.

Measurement of cytosolic free calcium concentration [Ca^{2+}]_i

[Ca^{2+}]_i was measured by resuspending the cells in HBS supplemented with glucose (10 mM), CaCl₂ (1 mM) and bovine serum albumin (1%, w/v), to a cell density of 6×10^6 cells ml⁻¹ (McCarthy *et al.*, 1989). The cell suspension was incubated with fura-2 acetoxymethyl ester (1 µM) for 30 min at 37°C, then the cells were washed and resuspended in the same medium to a cell density of 2×10^6 cells ml⁻¹. The cell suspension was kept at room temperature until required, and could be kept for at least 5 h after loading without substantial leakage of fura-2 from the cells, increase in basal [Ca^{2+}]_i, or decrease in responsiveness to FMLP. In all experiments, a control sample of cells was not incubated with dye; these cells were used to measure autofluorescence.

Samples (0.7 ml) of cell suspension were centrifuged in a Hettich microfuge for 30 s at 11,000 g and then resuspended to 1 ml with HBS containing glucose (10 mM) and CaCl₂ (1 mM). Fura-2 fluorescence of neutrophil suspensions (510 nm emission) was determined at 37°C with constant stirring, using a Hitachi F-4010 fluorescence spectrophotometer. [Ca^{2+}]_i was measured as the ratio of fura-2 fluorescence at excitation wavelengths of 340 and 380 nm according to the method of Grynkiewicz *et al.* (1985). Cells were incubated for 1 min before the addition of test drug (≤ 10 µl) which was followed after 2 min with FMLP (1 nM). The FMLP-induced change in [Ca^{2+}]_i was calculated as the maximal change in [Ca^{2+}]_i during the 30 s following addition of FMLP. Control responses to FMLP alone were tested regularly throughout the course of each experiment. The various concentrations of prostanoids tested were added in a random sequence. There were no detectable solvent effects for any of the compounds tested.

Measurement of [^3H]-cyclic AMP accumulation

Cyclic AMP production was assayed by measuring the conversion of [^3H]-ATP to [^3H]-cyclic AMP according to Barber *et al.* (1980). Rat neutrophils were incubated with [^3H]-adenine (10 µCi ml⁻¹) for 60 min at 37°C in buffer (HBS supplemented with glucose (10 mM), CaCl₂ (1 mM) and

bovine serum albumin (1%, w/v)) at a cell density of 3×10^7 cells ml⁻¹. Labelled cells were washed and incubated at 1×10^7 cells ml⁻¹ in buffer containing the type IV phosphodiesterase (PDE) inhibitor, rolipram (0.1 mM), indomethacin (3 µM) and adenosine deaminase (1 u ml⁻¹). After 10 min incubation at 37°C, test drugs were added for a further 10 min when the reaction was terminated by the addition of ice-cold 7.5% trichloroacetic acid.

[^3H]-ATP was separated from [^3H]-cyclic AMP by column chromatography according to Salomon *et al.* (1974). [^{14}C]-cyclic AMP (10,000 d.p.m.) was added to each sample prior to column elution to correct for the percentage recovery of the columns. The levels of [^3H]-cyclic AMP and [^{14}C]-cyclic AMP were determined by liquid scintillation counting. Results are expressed as either (1) relative to the basal activity represented by [^3H]-cyclic AMP generated in the absence of exogenous stimuli, or (2) relative to the response to cicaprost (0.1 µM).

Data analysis

EC₅₀ values (concentration of FMLP producing 50% of maximal response) and IC₅₀ values (concentration of test compound producing 50% inhibition of maximal response) were calculated using the four-parameter logistic curve fitting programme ALLFIT (De Lean *et al.*, 1978). Results are expressed as means with standard error of the mean of (n) experiments. Statistical analysis was performed using Student's unpaired t test.

Materials

FMLP, fura-2 acetoxymethyl ester, PGD₂, PGE₂, PGF_{2α}, neutral alumina (type WN-3), and imidazole were supplied by the Sigma Chemical Co. (U.S.A.). PGE₁, 17-phenyl-ω-trinor PGE₂ and U 46619 (15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid were purchased from Cayman Chemicals (U.S.A.). NIH thioglycollate broth was purchased from Difco (U.S.A.) and Ficoll-paque from Pharmacia (Sweden). 8-[^3H]-adenine (specific activity 27 Ci mmol⁻¹) and [adenine-U- ^{14}C]-cyclic AMP (specific activity 278 mCi mmol⁻¹) were purchased from Amersham International (U.K.). Dowex AG 50W-XA (200–400 mesh) was purchased from Bio-Rad (U.S.A.). The gifts of prostanoids are gratefully acknowledged: AH 13205 (*trans*-2-[4-(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoic acid) and GR 63799X (13-oxa-13,14-dihydro-16-phenoxy-ω-tetranor-PGE₂-4-(benzoylamino)phenyl ester; Glaxo, U.K.); cicaprost, iloprost, rolipram and sulprostone (Schering AG, Germany); butaprost (Bayer, U.K.); misoprostol (Searle, U.S.A.), and MB 28767 (*rac* 15S-hydroxy-9-oxo-16-phenoxy-ω-tetranor-prost-13E-enoic acid; Rhone-Poulenc, U.K.).

Results

FMLP-induced neutrophil aggregation

The addition of FMLP to suspensions of neutrophils produced a rapid concentration-dependent increase in aggregation which was maximal at 60 nM. The EC₅₀ value for FMLP was 18.0 ± 3.4 nM ($n = 5$).

Effect of prostanoids on FMLP-induced aggregation

When tested at 1 µM, none of the fourteen compounds tested had any direct effect of neutrophil aggregation. However, at the same concentration, PGE₁, PGE₂, butaprost, misoprostol, MB 28767, iloprost and cicaprost all significantly inhibited aggregation stimulated by FMLP (16 nM) (Table 1).

PGE₂ and cicaprost produced a concentration-dependent inhibition of FMLP-stimulated aggregation with maximal inhibition of approximately 50–60% (Figure 1). IC₅₀ values

for PGE₂ and cicaprost were 5.6 and 7.9 nM respectively. PGD₂, PGF_{2α}, and U 46619 had no effect on FMLP-mediated responses at 0.1 nM to 1 μM (Figure 1); sulprostone was similarly inactive (data not shown). Inclusion of indomethacin (3 μM) to inhibit any endogenous production of prostanoids had no effect on the concentration-response curve for PGE₂ (data not shown) and was therefore not routinely included in this series of experiments.

Effect of FMLP on rat neutrophil [Ca²⁺]_i

In the presence of external calcium (1 mM), FMLP caused a rapid, concentration-dependent increase in [Ca²⁺]_i which

Table 1 Effects of prostanoid agonists on the FMLP-stimulated responses of rat neutrophils

Prostanoid	Receptor specificity	Aggregation (% control)	[Ca ²⁺] _i rise (% control)
PGE ₂	EP	38 ± 4***	46 ± 5***
Cicaprost	IP	57 ± 1***	61 ± 5***
PGD ₂	DP	99 ± 6	100 ± 6
PGF _{2α}	FP	95 ± 12	98 ± 5
U 46619	TP	105 ± 4	99 ± 8
Iloprost	IP/EP ₁	58 ± 6***	56 ± 3***
PGE ₁	EP/IP	52 ± 5***	60 ± 8***
17-Phenyl-ω-trinor PGE ₂	EP ₁	86 ± 8	99 ± 5
AH 13205	EP ₂	101 ± 8	99 ± 3
Butaprost	EP ₂	73 ± 7***	78 ± 5*
Misoprostol	EP ₂ /EP ₃	48 ± 5***	63 ± 7**
GR 63799X	EP ₃	103 ± 5	87 ± 6
MB 28767	EP ₃ /TP	76 ± 7**	101 ± 3
Sulprostone	EP ₃ /EP ₁	101 ± 8	102 ± 4

Prostanoids (1 μM) were added 2 min before an EC₅₀ concentration of FMLP. Results are expressed as % control response (means ± s.e.mean, *n* = 4–7). For the aggregation experiments, the control response to FMLP (16 nM) was 16.3 ± 0.7 mm (*n* = 13). For the calcium experiments, the control response to FMLP (1 nM) was 126 ± 8 nM increase in [Ca²⁺]_i (*n* = 20). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared to FMLP alone.

Figure 1 The effects of prostanoid receptor agonists on FMLP-induced neutrophil aggregation. Rat neutrophils were incubated for 2 min with test compound prior to the addition of an EC₅₀ concentration of FMLP (16 nM). Control response to FMLP (16 nM) = 100 ± 6%, representing an aggregation response of 20.3 ± 0.7 mm (*n* = 13). Data shown are means with s.e.mean of 4 to 6 separate determinations: PGE₂ (●); cicaprost (○); U 46619 (■); PGD₂ (▲); PGF_{2α} (Δ).

decayed to about 40% of its peak value within 1 min, after which the decay was much slower (Figure 2). A maximally effective concentration of FMLP (10 nM) increased [Ca²⁺]_i by 297 ± 45 nM, from a basal level of 26 ± 1 nM, with an EC₅₀ of 1.17 ± 0.08 nM (*n* = 3).

Effect of prostanoid agonists on the FMLP [Ca²⁺]_i response

None of the fourteen compounds tested had any direct effect on rat neutrophil [Ca²⁺]_i over the concentration range 0.1 nM to 1 μM. However, several of the prostanoids tested influenced the subsequent response to a half-maximally effective concentration of FMLP (1 nM) added 2 min after the test compound (Figures 2 and 3).

At concentrations of 1 nM, or less, PGE₁, PGE₂, the PGI₂-mimetics, cicaprost and iloprost, butaprost (an EP₂-selective agonist) and misoprostol (an EP₂/EP₃-receptor agonist) tended to enhance the increase in [Ca²⁺]_i. However, this enhancement was relatively small and rarely reached the level of statistical significance. In contrast, at concentrations greater than 10 nM, PGE₁, PGE₂, cicaprost and iloprost all inhibited the response to FMLP with maximal inhibition at approximately 44% of the control response (*P* < 0.001 compared with the control response). The inhibitory phase of all four concentration-response curves were virtually superimposable, producing IC₅₀ values of 33, 18, 34 and 38 nM for PGE₂, cicaprost, PGE₁ and iloprost respectively.

Butaprost and misoprostol also produced a statistically significant inhibition of the FMLP-stimulated response at 1 μM (*P* < 0.05). All inhibitory activities correlated well with inhibition of aggregation (*r* = 0.885, *P* < 0.01) except that MB 28767 had no effect on FMLP-stimulated [Ca²⁺]_i (Table 1).

The concentration-response curves for the EP₃-selective agonists, GR 63799X and MB 28767, and the EP₃/EP₁-receptor agonist, sulprostone, tended to lie above the control response line, with the facilitation of the FMLP response by GR 63799X (1 nM) being statistically significant (*P* < 0.05). The thromboxane A₂-mimetic, U 46619, PGD₂, PGF_{2α} and the EP₂-receptor agonist, AH 13205, had no effect on the FMLP-induced increase in [Ca²⁺]_i. The EP₁-selective agonist 17-phenyl-ω-trinor PGE₂ (Lawrence & Jones, 1992) produced a slight increase in response at 1 nM whilst being without effect at all other concentrations tested.

Figure 2 The effect of PGE₂ on FMLP-induced increase in rat neutrophil [Ca²⁺]_i. Results shown are the typical responses of fura-2-loaded neutrophils to buffer (solid line) or PGE₂ (1 μM; dashed line) added at *t* = 1 min followed by FMLP (1 nM) at *t* = 3 min.

Figure 3 The effects of prostanoid receptor agonists on FMLP-induced increase in rat neutrophil $[Ca^{2+}]_i$. Control response to FMLP (1 nM) = $100 \pm 7\%$ ($n = 20$). Basal $[Ca^{2+}]_i$ was 21 ± 2 nM, and the increase in $[Ca^{2+}]_i$ due to FMLP (1 nM) was 126 ± 8 nM ($n = 20$). Data shown are means with s.e. mean of 5 to 7 separate determinations. (a) PGE₂ (●); cicaprost (○); U 46619 (■); PGD₂ (▲); (b) PGE₁ (●); iloprost (○); 17-phenyl- ω -trinor PGE₂ (▲); (c) GR 63799X (●); sulprostone (○); MB 28767 (▲); (d) Butaprost (●); misoprostol (▲); AH 13205 (○).

$[^3H]$ -cyclic AMP accumulation

PGE₂ and cicaprost produced a concentration-dependent increase in $[^3H]$ -cyclic AMP accumulation, with cicaprost being approximately 10 fold more potent than PGE₂ (Figure 4a). PGD₂, PGF_{2 α} , and U 46619 had no effect on adenylyl cyclase activity at 0.1 nM to 1 μ M (Figure 4a). Of the EP-receptor selective drugs tested, 17-phenyl- ω -trinor PGE₂ and sulprostone were without effect at 0.1 nM to 1 μ M, whilst butaprost produced a 2 fold increase in $[^3H]$ -cyclic AMP accumulation at 1 μ M (Figure 4b).

To determine if any of our selected compounds had EP₃-like activity, we incubated the cells with 1 μ M test drug in the presence and absence of an adenylyl cyclase stimulant to look for inhibition of adenylyl cyclase activity. Cicaprost (0.1 μ M) was chosen to activate adenylyl cyclase as it was considerably more effective ($237 \pm 25\%$ control, $n = 6$) than either forskolin (100 μ M, $133 \pm 27\%$ control, $n = 4$) or isoprenaline (1 μ M, $186 \pm 11\%$ control, $n = 4$). When tested alone, PGE₂, PGE₁, iloprost, butaprost and misoprostol all increased $[^3H]$ -cyclic AMP accumulation (Figure 5a,b), with the response to PGE₂, PGE₁ and iloprost being statistically significant ($P < 0.05$). None of the remaining eight compounds had any effect alone. GR 63799X, MB 28767 and sulprostone, i.e. the EP₃-receptor agonists, all failed to inhibit cicaprost-stimulated $[^3H]$ -cyclic AMP accumulation. Sulprostone was tested further at 0.1 nM to 1 μ M and had no effect on the response to cicaprost (data not shown). Similarly none of the remaining compounds tested at 1 μ M had any inhibitory effect on the response to cicaprost (Figure 5).

Discussion

In this investigation we have characterized the prostanoid receptors present on rat neutrophils by using a mixture of functional and biochemical assays, and a wide range of prostanoid agonists of varying receptor selectivity. The primary

response of neutrophils to activation by FMLP is the rapid elevation of $[Ca^{2+}]_i$ (McCarthy *et al.*, 1989) closely followed by adherence to, and migration across, the endothelial cell layer (Winder *et al.*, 1993). Neutrophils stimulated by chemotactic factors undergo a transient change in surface properties that permits the cells to adhere more readily to surfaces such as endothelial cells, and to each other (Hoffstein *et al.*, 1982). Neutrophil aggregation *in vitro* has therefore been used in the present study as a measure of the adhesion response.

Of the five major prostanoid agonists (PGE₂, cicaprost, PGD₂, PGF_{2 α} , and U 46619) that define the EP, IP, DP, FP and TP-receptors (Coleman *et al.*, 1990), only PGE₂ and cicaprost inhibited FMLP-stimulated elevation of $[Ca^{2+}]_i$ and aggregation. The log concentration-response curves for each agent in the two assays were parallel. Other prostaglandins with IP-agonist activity, i.e. PGE₁ and iloprost, were also similarly potent in inhibiting FMLP-stimulated elevation of $[Ca^{2+}]_i$ with IC₅₀ values of approximately 36 nM.

PGE₂ and IP-receptor agonists such as PGI₂ and beraprost have also been reported to be equipotent in inhibiting FMLP-induced chemotaxis in rat neutrophils (Kainoh *et al.*, 1990). Cicaprost is considerably more specific than iloprost as an IP-receptor agonist (Dong *et al.*, 1986; Armstrong *et al.*, 1989), and its profile in our experiments indicates the presence of IP-receptors on the rat neutrophil. Our observations therefore confirm the presence of both EP and IP-receptors capable of inhibiting rat neutrophil function and contrast with the relative lack of potency of IP-receptor agonists in assays of human neutrophil function (Hecker *et al.*, 1990; Wheeldon & Vardey, 1993). Our data also confirm the observations of Ham *et al.* (1983) who noted that rat neutrophils were unresponsive to PGD₂, which was in marked contrast to the high potency of DP-receptor agonists in inhibiting opsonized zymosan-induced release of LTB₄ from human neutrophils (Wheeldon & Vardey, 1993).

In this present paper we show that at the level of the initial response to FMLP, i.e. the elevation of $[Ca^{2+}]_i$, prostaglandin

Figure 4 The effects of prostanoid receptor agonists on $[^3\text{H}]$ -cyclic AMP accumulation in the presence of rolipram (0.1 mM), indomethacin (3 μM) and adenosine deaminase (1 U ml^{-1}). Rat neutrophils were incubated for 10 min before the addition of test compound for a further 10 min. Data represent the means with s.e. mean of 3 experiments performed in triplicate. (a) PGE_2 (●); cicaprost (○); U 46619 (■); PGD_2 (▲); $\text{PGF}_{2\alpha}$ (△); (b) Butaprost (●); 17-phenyl- ω -trinor PGE_2 (▲); sulprostone (○).

Figure 5 The effects of prostanoid receptor agonists on $[^3\text{H}]$ -cyclic AMP accumulation stimulated by cicaprost. Rat neutrophils were incubated in the presence of rolipram (0.1 mM), indomethacin (3 μM) and adenosine deaminase (1 U ml^{-1}) for 10 min before the addition of test compound (1 μM), in the presence (hatched columns) and absence (open columns) of cicaprost (0.1 μM). The reaction was stopped after a further 10 min. Data represent the means with s.e. mean of 3 experiments performed in triplicate. Cicaprost (0.1 μM) elevated $[^3\text{H}]$ -cyclic AMP levels to $223 \pm 38\%$ control ($n = 3$). Abbreviations: Con (control), 17- PGE_2 (17-phenol- ω -trinor PGE_2), AH (AH13205), But (butaprost), Miso (misoprostol), GR (GR 63799X), MB (MB 28767), Sulp (sulprostone) and Ilo (iloprost).

analogues active at the EP_2 -receptor, e.g. PGE_1 , PGE_2 , butaprost and misoprostol, all have an inhibitory action. This is unlikely to be due to any cytotoxic effect since there was no obvious fura-2 loss from the cells on the addition of prostanoids. These same compounds, tested at 1 μM , also inhibited the aggregation response to FMLP, thus identifying an inhibitory role for EP_2 -receptors in rat neutrophils. MB 28767 was the only compound tested which inhibited FMLP-stimulated aggregation yet had no effect on FMLP-stimulated $[\text{Ca}^{2+}]_i$. MB 28767 has previously been shown to exhibit EP_2 -like activity by relaxing rabbit jugular vein with an equi-effective molar ratio of approximately 400 when compared with PGE_2 (Lawrence & Jones, 1992). Therefore it is probable that we are able to detect an EP_2 -like effect of MB 28767 due to the relatively high sensitivity of the aggregation assay to PGE_2 (IC_{50} 5.6 nM) when compared with the sensitivity of the calcium assay to PGE_2 (IC_{50} 33 nM).

In the same study by Lawrence & Jones (1992), the relaxant potency of butaprost on the rabbit jugular vein was found to be surprisingly weak (about 700 and 80 times less than PGE_2 and misoprostol respectively) and this led to the first suggestion of the existence of subtypes of the EP_2 -receptor. In our present study, the EP_2 -selective agonist AH 13205 (Nias *et al.*, 1993) at 1 μM had no effect on FMLP-mediated activation of the rat neutrophil or on the accumulation of $[^3\text{H}]$ -cyclic AMP. In contrast, AH 13205 was only 16 fold less potent than PGE_2 in inhibiting opsonized zymosan-induced release of LTB_4 from human neutrophils (Wheeldon & Vardey, 1993). More recently AH 13205 has been used to distinguish the EP_2 -receptor from a recently

identified subtype, the EP_4 -receptor, present in pig saphenous vein where AH 13205 was reported to be 11,000 fold less potent than PGE_2 (Coleman *et al.*, 1994). It is conceivable that AH 13205 may have shown an effect in our study at a higher concentration, but we chose to limit the concentration of compounds tested to 1 μM in order to maintain their specificity of action. Since in the present study the potency of butaprost is much closer to that of misoprostol, it is probable that our results support the presence of conventional EP_2 -receptors.

The inhibitory action of prostaglandins on neutrophils is considered to be due to stimulation of cyclic AMP production since chemoattractants such as FMLP indirectly elevate intracellular cyclic AMP levels as part of a negative regulatory system (Verghese *et al.*, 1985), and prostaglandin-induced inhibition of neutrophil function tends to be paralleled by increases in cyclic AMP (Ney & Schrör, 1991). We have found that only those compounds which stimulated adenylyl cyclase activity were able to inhibit FMLP-stimulated $[\text{Ca}^{2+}]_i$ and neutrophil aggregation. Whilst the drugs which can elevate neutrophil $[^3\text{H}]$ -cyclic AMP clearly inhibit neutrophil activity and might be considered as anti-inflammatory, one rarely observes more than 60% inhibition of any measure of neutrophil function (see also Wheeldon & Vardey, 1993). Clearly there are important regulators of neutrophil function other than adenylyl cyclase.

The lack of effect of prostaglandins alone in stimulating an increase in $[\text{Ca}^{2+}]_i$ and aggregation also suggests the lack of

prostaglandin receptors directly coupled to phospholipase C. In particular, the lack of effect of the EP₁-selective agonist 17-phenyl- ω -trilor PGE₂ would indicate the absence of EP₁-receptors on rat peritoneal neutrophils.

Prostanoids with EP₃-like activity such as PGE₁, PGE₂, GR 63799X, MB 28767, misoprostol and sulprostone all tended to enhance the increase in [Ca²⁺]_i due to FMLP, although rarely by more than 20% of the control response in the absence of prostanoid. Although the apparent potentiation of FMLP-mediated increases in [Ca²⁺]_i by various prostanoids is relatively small and rarely reached a level of statistical significance, the response could not be attributed to an artifact of the experimental design and was of a similar magnitude as the potentiation by sulprostone (1 μ M) of opsonized zymosan-induced release of LTB₄ from human neutrophils reported by Wheeldon & Vardey (1993). Any potentiation of FMLP-mediated increases in [Ca²⁺]_i might be expected to result in an enhanced aggregatory response to FMLP. However, sulprostone (0.1 nM to 1 μ M) had no potentiating effect on FMLP-mediated neutrophil aggregation. Activation of EP₃-receptors would also be expected to inhibit adenyl cyclase activity. GR 63799X, MB 28767 or sulprostone did not inhibit cicaprost-stimulated [³H]-cyclic

AMP accumulation, thus providing further evidence for the lack of EP₃-receptors on rat neutrophils. It is not clear at present why low concentrations of EP₃ and some EP₂-receptor agonists appeared to facilitate FMLP-mediated increase in [Ca²⁺]_i. The absence of specific receptor antagonists clearly hampers the interpretation of the results here.

In conclusion, we have shown that the ability of FMLP to increase [Ca²⁺]_i and to stimulate an aggregation response in rat neutrophils is not affected by DP, FP or TP-receptor agonists, but can be inhibited by EP and IP-receptors agonists. The lack of DP-receptors and the presence of functionally active IP-receptors contrast with reported studies on human neutrophils. Rat neutrophils appear to lack EP₁-receptors whilst possessing an EP₂ or EP₂-like receptor which appears to mediate the inhibitory effect of PGE₂. We have found no evidence for an EP₃-receptor capable of facilitating the chemotactic responses of rat neutrophils.

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