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Characterization of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils

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1 The aims of this study were to characterize the EP receptor subtype mediating the inhibition of superoxide anion generation by formyl methionyl leucine phenylalanine (FMLP)-stimulated human neutrophils, and to test the hypothesis that adenosine 3':5'-cyclic monophosphate (cyclic AMP) is the second messenger mediating the inhibition of the neutrophil by prostaglandin (PG)E_2.

2 PGE_2 (0.001–10 μM) inhibited FMLP (100 nM)-induced O_2-generation from human peripheral blood neutrophils in a concentration-dependent manner, with an EC_{50} of 0.15 ± 0.03 μM, and a maximum effect ranging from 36–84% (mean inhibition of 68.7 ± 2.5%, $n = 32$).

3 The EP_2-receptor agonists, misoprostol, 11-deoxy PGE_1, AH13205 and butaprost, all at 10 μM, inhibited O_2^- generation, causing 95.5 ± 2.9%, 56.8 ± 5.2%, 37.1 ± 6.6% and 18.9 ± 4.4% inhibition respectively, the latter two being much less effective than PGE_2. Similarly, the EP_1-receptor agonist, 17-phenyl PGE_2 (10 μM), and the EP_3/EP_1-receptor agonist, sulprostone (10 μM), also inhibited O_2^- generation, causing 32.2 ± 7.0% and 15.3 ± 3.4% inhibition respectively.

4 The non-selective phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX, 0.25 mM) inhibited the FMLP response by 54.5 ± 5.0%. In addition, IBMX shifted concentration-effect curves for PGE_2, misoprostol, 11-deoxy PGE_1, butaprost, and AH 13205 to the left, to give EC_{50}s of 0.04 ± 0.03 ($n = 13$), 0.07 ± 0.03 ($n = 4$), 0.08 ± 0.03 ($n = 4$), 0.33 ± 0.13 ($n = 4$) and 0.41 ± 0.2 μM ($n = 3$) respectively, allowing equieffective concentration-ratios (EECs, $PGE_2 = 1$) of 11.5, 5.3, 50.7 and 12.7 to be calculated. This agrees well with the relative potencies of these agonists at EP_2 receptors.

5 By contrast, even in the presence of IBMX (0.25 mM), sulprostone and 17-phenyl PGE_2 were only effective at the highest concentration (10 μM), and gave EECs of > 700 and 486 respectively, suggesting that EP_1 or EP_3 receptors are not involved.

6 The selective type IV phosphodiesterase inhibitor, rolipram at 2 and 10 nM did not inhibit the FMLP response, but at the higher concentration of 50 nM, it decreased the FMLP response by 46.6 ± 7.3%. However, rolipram shifted concentration-effect curves for PGE_2 to the left to give EC_{50}s of 0.06 ± 0.022, 0.015 ± 0.0, 0.012 ± 0.006 μM at 2, 10 and 50 nM respectively, compared to the control EC_{50} of 0.27 ± 0.09 μM for PGE_2.

7 The EP_4/TP receptor blocking drug, AH 23848B (10 μM, 10 min) did not inhibit O_2^- generation by PGE_2, but was found to potentiate significantly the effect of PGE_2 at the lower concentrations of PGE_2 tested (0.001–0.1 μM).

8 The adenylate cyclase inhibitor, SQ 22,536 (0.1 mM, 2 min) reduced PGE_2-induced inhibition of O_2^- production, giving an EC_{50} in the absence of SQ 22,536 of 0.24 ± 0.1, and 1.9 ± 1.1 μM in its presence.

9 These results suggest that inhibition of superoxide generation by PGE_2 is mediated by stimulation of EP_2 receptors and activation of adenylate cyclase, leading to the elevation of intracellular levels of cyclic AMP.

Keywords: EP receptors; neutrophils; cyclic AMP; superoxide anion generation

Introduction

The main function of neutrophils is in host-defence. Neutrophils ingest microorganisms by phagocytosis and release toxic substances such as superoxide (O_2^-) and lysosomal enzymes, which enable the neutrophil to kill any invading pathogen.

It has been shown that prostaglandin E_2, through activation of EP receptors, inhibits the generation of O_2^- by activated human neutrophils (Wheeldon & Vardey, 1993). The main aim of the present study was to determine the subtype of EP receptor involved, the likely candidates being EP_2 and EP_4 subtypes, both of which have been linked to stimulation of adenylate cyclase (Coleman *et al.*, 1994; Milne *et al.*, 1994). Thus, we also set out to test the hypothesis that adenosine 3': 5'-cyclic monophosphate (cyclic AMP) is the second messenger mediating inhibition of neutrophil activation.

For these purposes, the inhibitory effects of selective EP agonists, and the antagonistic effect of selective EP receptor blocking drugs have been evaluated. In addition, the modulatory effects of the adenylate cyclase inhibitor, SQ 22,536 (Harris *et al.*, 1979), the non-selective phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX), and the selective type IV phophodiesterase inhibitor, rolipram (Schudt *et al.*, 1991), have been assessed.

Furthermore, as it has previously been shown that adenosine released from neutrophils can inhibit O_2^- generation (Cronstein *et al.*, 1988), we have investigated the modulatory role of endogenous adenosine using adenosine deaminase (ADA) to break down adenosine (Cronstein *et al.*, 1984), and the non-selective A_1/A_2 antagonist, 8-phenyltheophylline (8-PT), to block the effects of released adenosine (see Collis & Hourani, 1993).

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Methods

Isolation of human neutrophils

Venous blood was taken from the forearm of healthy volunteers, and anti-coagulated with heparin 10 u ml^{-1} of blood, mixed with an equal volume of 3% dextran in 0.9% w/v saline and left to stand for 45 min. The leukocyte-rich plasma was then removed and centrifuged at 280 *g* for 10 min and the resultant pellet resuspended in 55% Percoll (Pharmacia) and layered on top of a discontinuous Percoll gradient (3 ml 70% Percoll layered on top of 5 ml 81% Percoll). The tubes were then centrifuged for 30 min at 650 *g* to separate neutrophils from mononuclear cells. The neutrophils (lower layer) were removed and washed twice. Red cells remaining in the neutrophil pellet were lysed by resuspending the pellet in 10 ml ice cold 0.2% w/v NaCl solution for 20 s, after which 10 ml of ice cold 1.6% w/v NaCl was added to return the cells to isotonic conditions. Cells were then counted in an improved Neubauer counting chamber, and their viability assessed by trypan blue exclusion. Finally the resultant pellet was resuspended at a concentration of 1.5×10^6 cells ml^{-1} in phosphate buffered saline (PBS containing Ca^{2+}/Mg^{2+}, Sigma), and kept at room temperature.

Superoxide anion production

Superoxide generation was assayed by spectrophotometric evaluation of the reduction of ferricytochrome C to ferrocytochrome C (A550nm).

Control tubes contained 5 μg per ml of cytochalasin B (used to enhance the amount of superoxide anion released during the reaction), 0.1 mg ml^{-1} cytochrome C and 90 u ml^{-1} superoxide dismutase (SOD). In sample tubes SOD was replaced with PBS. Cells (900 μl) were incubated with PBS or inhibitory agent in a gently shaking water bath for 10 min at 37°C prior to the addition of formyl methionyl leucine phenylalanine (FMLP) (100 nM). When antagonists were studied, they were incubated for 10 min prior to the addition of EP agonists or the adenosine mimetic, 5'-N-ethylcarboxamido-adenosine (NECA). Ten min after the addition of FMLP, the reaction was terminated by immersing the tubes in ice for 5 min and the samples centrifuged at 1000 r.p.m., 4°C, for 5 min, to sediment the cells.

The absorbance of the supernatants was measured with a Cary spectrophotometer.

Data analysis

Each incubation was carried out in duplicate and the values averaged. The amount of superoxide anion produced (nmol per 10^6 neutrophils per 10 min) was calculated with the following equation:

$$\frac{dE}{Q \times d} \times 10^6 \times \frac{1}{L}$$

$L = 1.5 \times 10^6$ cells per ml = concentration of human neutrophils; dE = (absorbance of samples without SOD)-(absorbance of samples with SOD); Q = coefficient of molar extinction $21.1 \times 10^3\ M^{-1}\ cm^{-1}$; d = thickness of cuvette = 1 cm.

Effects of the various drugs on O_2^- generation were expressed as the percentage inhibition of the response produced by a submaximally effective concentration of FMLP (100 nM).

EC_{50} values were calculated (concentration of agonist required to produce 50% of the maximal effect of PGE_2) for all the EP-receptor agonists, in the presence and absence of antagonists using the Apple Macintosh programme 'Kaleidagraph'. Kaleidagraph is not a graph-fitting but a graph-drawing programme and so uses the experimental maximum observed to determine the EC_{50} value. A control curve for PGE_2 was run in each neutrophil preparation, so that the EC_{50} values for other agonists could be determined relative to the maximum effect achieved with PGE_2. From EC_{50} values, equieffective concentration ratios (EECs) were calculated relative to the standard agonist, PGE_2 (EEC = 1).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (s.e.mean), of the averaged result taken from a minimum of four separate experiments. Data were analysed using Student's paired or unpaired 2 tailed *t* test as appropriate.

Materials

The following compounds were gifts which we gratefully acknowledge: sulprostone and rolipram from Dr E Schillinger, Schering AG, Berlin; butaprost from Dr P. Gardiner, Bayer, U.K.; AH 13205 (*trans*-2[4-(1-hydroxyhexyl)pentyl) phenyl]-5-oxocyclo pentaneheptanoic acid), AH 6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid) and AH 23848B 9[1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxyl]-2-(4-morpho-linyl) -3-oxocyclopentyl]-4-heptanoic acid) from Dr B. Bain, Glaxo, U.K.; 17-phenyl-ω-trinor PGE_2 and 11-deoxy PGE_1 were purchased from Cayman Chemicals, U.S.A.; SQ 22,536 ((9-tetrahydro-2-furyl)adenine) was synthesised in the department by Dr N.H. Wilson.

Isobutyl methylxanthine (IBMX), FMLP, ferricytochrome C and superoxide dismutase (SOD) were purchased from Sigma; Cytochalasin B from Aldrich; 5'-N-ethylcarboxamido-adenosine (NECA) from R.B.I. Ethanolic stock solutions of the prostanoids (10–30 mM) were stored at −20°C.

Results

Inhibition of superoxide generation by PGE_2

FMLP (100 nM) in the presence of cytochalasin B (5 μg per ml), induced a submaximal release of O_2^- of 6.7 ± 2.7 nmol per 10^6 cells per 10 min ($n = 6$). This concentration of FMLP achieved 91.9 ± 6.0% of the maximal effect induced by 300 nM FMLP, $n = 5$. PGE_2 (0.001–10 μM) produced a concentration-related inhibition of FMLP-induced O_2^- generation, achieving a maximal effect of 68.7 ± 2.5% inhibition at a concentration of 10 μM ($EC_{50} = 0.15 \pm 0.03$ μM, $n = 32$). The maximal degree of inhibition was variable ranging from 36–84%. A 10 min pre-incubation with PGE_2 prior to the addition of FMLP was selected as consistent with studies published by other workers. Pre-incubation times of 0, 15 and 30 min gave EC_{50}s for PGE_2 of 0.15 ± 0.07, 0.17 ± 0.07 and 0.11 ± 0.06 μM respectively ($n = 4$), none of which varied significantly ($P > 0.05$) from that at 10 min.

Inhibition of superoxide generation by selective EP agonists

The selective EP_2-receptor agonists, 11-deoxy PGE_1, AH13205 and butaprost, (all at 10 μM) were less potent and caused significantly less inhibition ($P < 0.05$) than PGE_2, causing 49.3 ± 5.4% ($n = 6$), 32.1 ± 3.4% ($n = 8$) and 13.9 ± 4.3% ($n = 16$) inhibition respectively at concentrations of 10 μM (Figure 1a). Taking the maximal effect of PGE_2 at 10 μM as 100% response, apparent EC_{50}s are 0.5 ± 0.2 ($n = 6$) and ⩾ 10.0 ($n = 8$) and > 10.0 μM ($n = 16$) respectively. Misoprostol, an EP_2/EP_3-receptor agonist, was slightly less potent than PGE_2 ($EC_{50} = 0.35 \pm 0.1$ μM ($n = 4$), with an EEC of 2.8), but more effective than PGE_2 at 10 μM, giving a maximal inhibition of 95.5 ± 2.9% (Figure 1a).

The cyclo-oxygenase inhibitor, indomethacin (3 μM) did not significantly effect inhibition of superoxide generation by PGE_2, butaprost or AH 13205 ($n = 4$) (Figure 1b).

The EP_1 receptor agonist, 17-phenyl-ω-trinor PGE_2 (0.1–10 μM) caused significantly ($P<0.05$) less inhibition than PGE_2 giving 32.2 ± 7.1% inhibition at a concentration of 10 μM (EC_{50} = 16.9 ± 8.4 μM, n = 4) (Figure 1c). Similarly, the EP_1/EP_3 receptor agonist sulprostone (0.1–10 μM) gave a maximal inhibition of only 15.3 ± 3.4% at 10 μM (EC_{25} = 17.7 ± 10.8 μM, n = 6), (Figure 1c).

Effect of IBMX on inhibitory effects of EP agonists

In the presence of IBMX (0.25 mM), concentration-effect curves for PGE_2 were shifted to the left (EC_{50} 0.04 ± 0.03 μM, n = 13), and the maximum inhibition at 10 μM increased to 83.9 ± 5.2%. IBMX (0.25 mM) itself reduced the FMLP response by 54.9 ± 5.0%, and % inhibitions in the presence of EP agonists were calculated from this reduced FMLP response.

The selective EP_2 receptor agonists, misoprostol, 11-deoxy PGE_1, AH 13205 and butaprost were also more effective inhibitors of O_2^- production by FMLP when the neutrophils were treated with IBMX (0.25 mM), causing 93.4 ± 3.5% inhibition at 10 μM (EC_{50} = 0.07 ± 0.03 μM, n = 4), 79.9 ± 9.7% (EC_{50} = 0.08 ± 0.03 μM, n = 4), 61.4 ± 6.0% at 10 μM (EC_{50} = 0.3 ± 0.1 μM, n = 4) and 57.1 ± 6.1% at 10 μM (EC_{50} = 0.4 ± 0.2, n = 4) respectively (Figure 2a). Thus, equieffective concentration-ratios (EECs) for misoprostol, 11-deoxy PGE_1, AH 13205 and butaprost of 1.75, 5.3, 12.7 and 50.7, respectively were determined.

In contrast, no significant difference ($P>0.05$) was observed with sulprostone in the presence of IBMX, whereas inhibition obtained with 17-phenyl PGE_2 was increased, but only at the highest concentration (10 μM, 56.8 ± 8.2% compared to 31.0 ± 6.0%) (Figure 2b).

Effect of selective prostanoid receptor antagonists

When neutrophils were pre-incubated with the EP_4 receptor antagonists, AH 23848B (10 μM, 10 min), there was no significant ($P>0.05$) antagonism of the inhibition produced by PGE_2. However, a potentiation of the inhibitory effect of PGE_2 at the lower concentrations of PGE_2 tested (0.001–0.1 μM) was observed (Figure 3a). This potentiation of inhibition by AH 23848B was also observed in the presence of IBMX (data not shown). An even greater potentiation of inhibition induced by butaprost (0.1–10 μM) was found (Figure 3b), although no inhibitory effect was observed with AH 23848B alone. Indeed, AH 23848B (10 μM, 10 min) increased the release of O_2^- stimulated by FMLP from

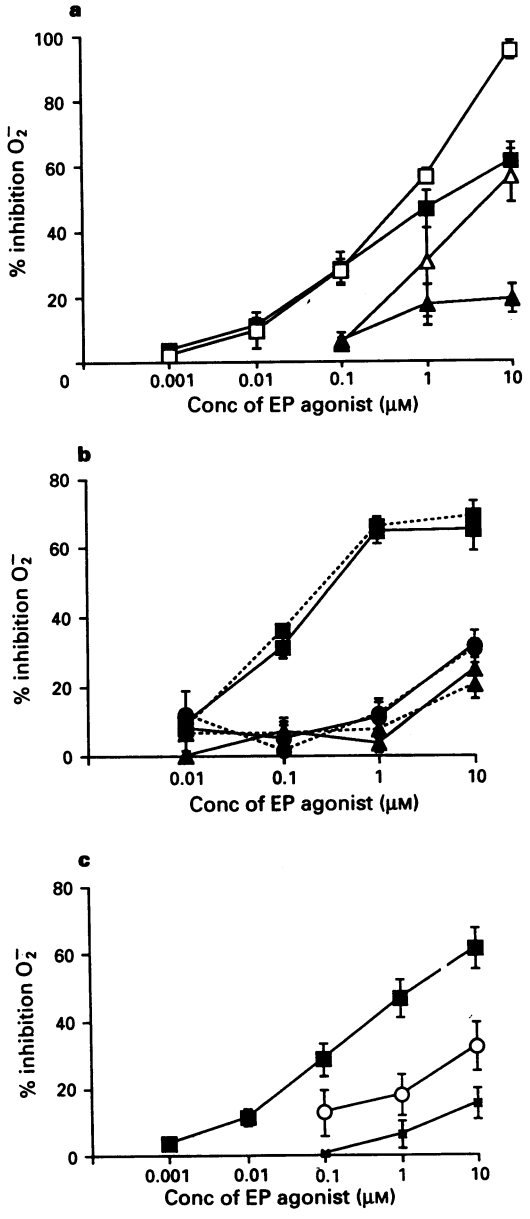

Figure 1 Log concentration-effect curves for EP-receptor agonist-inhibition of superoxide anion generation in human neutrophils induced by FMLP (100 nM). (a) PGE_2 (■), misoprostol (□), 11-deoxy-PGE_1 (△) and butaprost (▲); (b) PGE_2 (■), AH 13205 (●) and butaprost (▲). Continuous lines represent control neutrophils and dash lines, neutrohils treated with indomethacin (3 μM); (c) PGE_2 (■), 17-phenyl-ω-PGE_2 (○) and sulprostone (×). The values are the mean ± s.e.mean of 14 different donors for PGE_2, and 4 different donors for the other agonists. For abbreviations for this and all legends, see text.

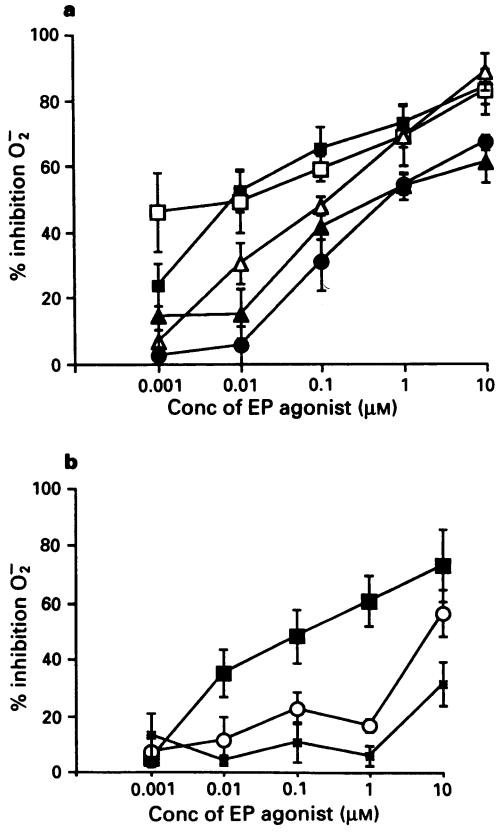

Figure 2 Log concentration-effect curves for EP-receptor agonist-inhibition of superoxide anion generation in neutrophils treated with IBMX (0.25 mM). (a) PGE_2 (■), misoprostol (□), 11-deoxy-PGE_1 (△), AH 13205 (●) and butaprost (▲); (b) PGE_2 (■), 17-phenyl-ω-PGE_2 (○) and sulprostone (×). The values are the mean ± s.e.mean of 4 different donors.

13.9 ± 1.4 to 16.0 ± 1.7 nmol O_2^- per 10 min per 10^6 cells, ($n = 5$). AH 23848B similarly potentiated inhibition induced by low concentrations of the adenosine-mimetic, 5'-N-ethylcarboxamido-adenosine (NECA) (Figure 3c).

The EP_1/DP-receptor antagonist, AH 6809 (10 μM, 10 min), significantly ($P < 0.05$) antagonized the inhibitory effects of 1.0 and 10, but not 0.1 μM PGE_2 (Figure 4a). From EC_{50} values, a pA_2 of 6.04 was calculated for AH 6809. AH 6809 on its own at 1 and 10 μM significantly ($P = 0.004$ and 0.02, respectively), increased O_2^- release induced by FMLP from 13.3 ± 2.4 to 15.9 ± 2.5 and 17.0 ± 2.0 nmol O_2^- per 10 min per 10^6 cells respectively ($n = 4$). AH 6809 also antagonized the inhibitory response induced by NECA (Figure 4b), with a pA_2 of 6.83.

Role of cyclic AMP in the inhibition of neutrophil activation

The adenylate cyclase inhibitor, SQ 22,536 (0.1 mM, 2 min), significantly ($P < 0.05$) reduced PGE_2-induced inhibition of O_2^- production, giving an EC_{50} in the absence and presence of SQ 22536 of 0.24 ± 0.1, and 1.9 ± 1.1 μM respectively (Figure 5). Conversely, non-selective inhibition of phosphodiesterase with IBMX (0.25 mM) potentiated the inhibitory effects of PGE_2 (Figure 2a). However, at this concentration, IBMX itself inhibited the FMLP response by 54.9 ± 5.0% ($n = 10$), a fact which could account for the increased effectiveness of PGE_2.

The specific type IV phosphodiesterase inhibitor, rolipram at concentrations of 2 and 10 nM, did not have any significant effect on FMLP response, reducing the FMLP effect by 8.8 ± 5.4% and 29.7 ± 15.1% respectively, whereas at the concentration of 50 nM, it significantly ($P < 0.05$) reduced the FMLP response, by 46.6 ± 8.4%. Yet rolipram, at all three concentrations (2, 10 and 50 nM) shifted the concentration-effect curve for PGE_2 leftwards, with EC_{50}s of 0.06 ± 0.02, 0.01 ± 0.0 and 0.012 ± 0.006 μM respectively (Figure 6). The cyclic AMP analogue, 8 bromo cyclic AMP (1.0–100 μM), did not give consistent results, inhibition varying from 0 to 14.5%.

Role of endogenously produced adenosine

Removal of endogenous adenosine with the enzyme, adenosine deaminase (ADA, 1.0 u ml^{-1}), had no significant effect upon concentration-effect curve for PGE_2 (EC_{50} of 0.42 ± 0.15 μM for PGE_2 and 0.1 ± 0.4 μM for PGE_2 + ADA) (Figure 7a), although the maximal inhibition was significantly increased from 55.3 ± 8.1 to 68.3 ± 6.8%, $n = 6$. ADA itself significantly ($P < 0.05$) increased the production of O_2^- by FMLP from $1.0 \times 10^{-8} \pm 0.2 \times 10^{-8}$ to $1.3 \times 10^{-8} \pm 0.1 \times 10^{-8}$ mol O_2^- per 10 min per 10^6 cells, $n = 6$. The non-selective adenosine receptor antagonist, 8-phenyl theophylline (8-PT, 10 μM), had no significant ($P > 0.05$) effect upon concentration-effect curves for PGE_2 (EC_{50}s of 0.4 ± 0.17 for

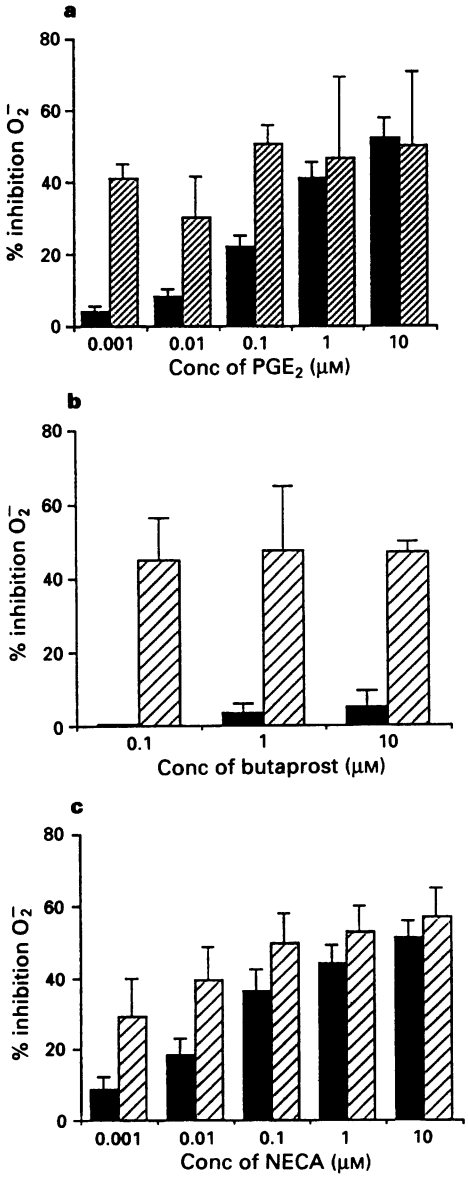

Figure 3 Effect of a 10 min pre-incubation with the EP_4 receptor anatagonist AH 23,848 (10 μM) on inhibition of FMLP-induced superoxide anion generation in human neutrophils by (a) PGE_2, (b) butaprost and (c) NECA. Solid columns represent control neutrophils and hatched columns, neutrophils treated with AH 23,848. The values are the mean ± s.e.mean of 4 different donors.

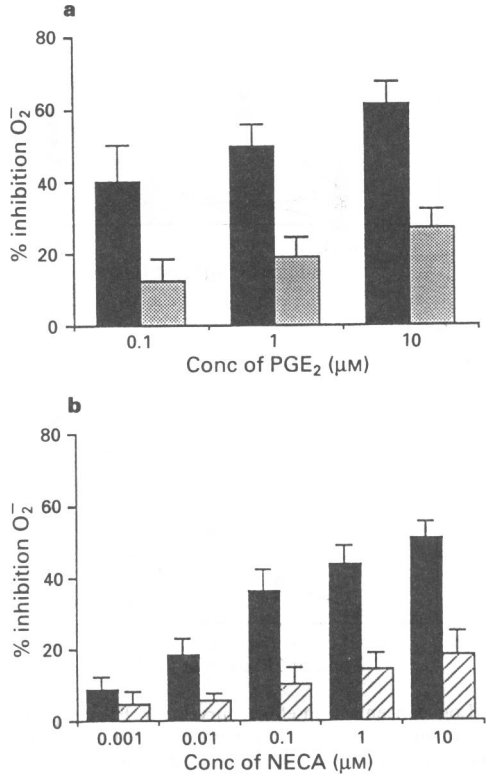

Figure 4 Effect of a 10 min pre-incubation with the EP_1 receptor anatagonist, AH 6809 (10 μM) on inhibition of FMLP-induced superoxide anion generation in human neutrophils by (a) PGE_2 and (b) NECA. Solid columns represent control neutrophils and hatched columns, neutrophils treated with AH 6809. The values are the mean ± s.e.mean of 4 different donors.

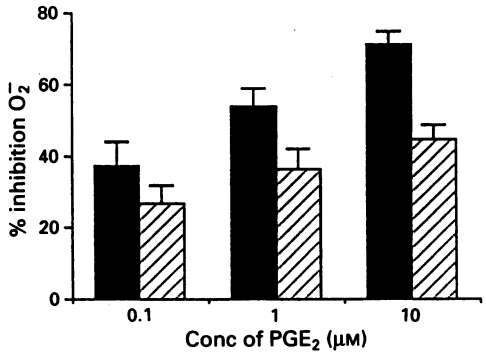

Figure 5 Effect of a 2 min pre-incubation with the adenylate cyclase inhibitor, SQ 22,536 (100 μM) on inhibition of FMLP-induced superoxide anion generation in human neutrophils by PGE_2. Solid columns represent control neutrophils and hatched columns, neutrophils treated with SQ 22,536. The values are the mean ± s.e.mean of 8 different donors.

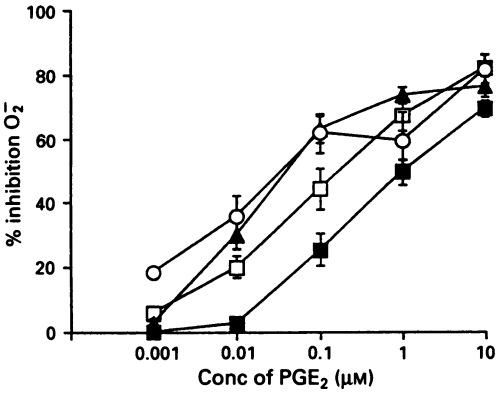

Figure 6 Log concentration-effect curves for inhibition of superoxide anion generation by PGE_2 in neutrophils treated with rolipram at (■) 0, (□) 2, (▲) 10 and (○) 50 nM. The values are the mean ± s.e.mean of 4 different donors.

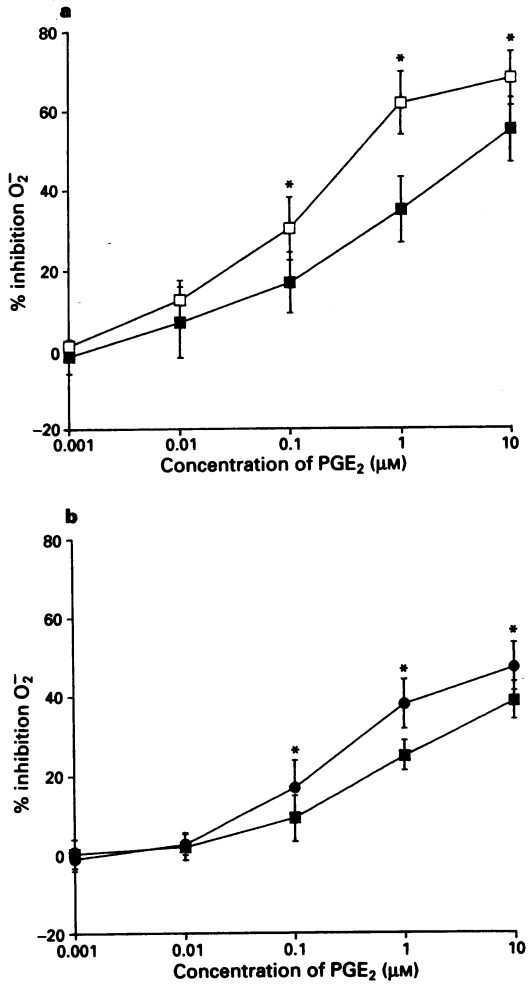

Figure 7 Log concentration-effect curves for inhibition of superoxide anion generation by PGE_2 in FMLP-stimulated human neutrophils. (a) PGE_2 in control neutrophils (■) and neutrophils treated with adenosine deaminase (1 u ml^{-1}) (□); (b) PGE_2 in control neutrophils (■) and neutrophils treated with 8-phenyl theophylline (10 μM) (●). * represents $P<0.05$. The values are the mean ± s.e.mean of a result from 6 and 5 donors respectively.

PGE_2 and 0.24 ± 0.1 μM for PGE_2 + 8-PT, $n = 5$) although the maximal response was increased slightly from 38.9 ± 4.9% to 47.7 ± 6.0% with 8-PT (Figure 7b).

Discussion

PGD_2 and PGE_2 are known to be potent inhibitors of O_2^- release in the human neutrophil (Gryglewski *et al.*, 1987; Wheeldon & Vardey, 1993). In the present study we have investigated the effects of selective EP receptor agonists and prostanoid receptor antagonists on FMLP-induced release of O_2^-, in order to elucidate the subtype of EP receptor occupied by PGE_2 in producing inhibition of O_2^- release.

The EP_2 receptor agonists, misoprostol, 11-deoxy PGE_1, butaprost (Gardiner, 1986; Lawrence & Jones, 1992) and AH 13205 (Nials *et al.*, 1993), while effective, were found to be less potent than PGE_2 itself, the latter two analogues producing a significant effect only at a concentration of 10 μM. However, one must question the selectivity of a compound at 10 μM, and the EP_1 agonist, 17-phenyl PGE_2 as well as the EP_1/EP_3 agonist, sulprostone, also showed weak inhibitory effects at this concentration, suggesting some crossover on to this inhibitory EP receptor at high concentrations. When the experiments were repeated with neutrophils treated with IBMX (0.25 mM), the concentration-effect curves for all the EP_2 agonists were significantly shifted to the left, allowing EECs of 11.5, 5.3, 50.7 and 12.7 to be calculated. This agrees well with the relative potency of these agonists on EP_2 receptor preparations (Nials *et al.*, 1991; 1993) where EECs of 1-4, 2-5, 6-30 and 30-100 have been reported.

In contrast, IBMX treatment did not significantly affect inhibition by sulprostone, and increased the activity of 17-phenyl PGE_2, only at the highest concentration 10 μM, allowing EECs of >700 and 486 to be calculated for sulprostone and 17-phenyl PGE_2 respectively. Sulprostone is a potent agonist at EP_3 receptors of the guinea-pig vas deferens, EEC = 0.15, PGE_2 = 1 (Coleman *et al.*, 1987) as well as at EP_1 receptors of the guinea-pig fundus, EEC = 2.5, PGE_2 = 1 (Coleman *et al.*, 1988) but shows weak activity at EP_2 receptors of the cat trachea, EEC > 7000 (Coleman *et al.*, 1988) or EP_4 receptors of the pig saphenous vein, EEC > 3000 (Coleman *et al.*, 1994). Similarly, 17-phenyl PGE_2 is a potent agonist at EP_1 receptors, but not at EP_2 receptors (Lawrence *et al.*, 1992). This apparent lack of activity by these agents, therefore, confirms that the EP receptor of major importance in mediating PGE_2-induced inhibition of O_2^--release is not of the EP_1 or EP_3 receptor subtypes.

The results so far imply that either EP_2 or EP_4 receptors are those involved in PGE_2-induced inhibition of O_2^- production. Investigation of the EP_4 receptor of the pig saphenous vein (Milne *et al.*, 1994) found 11-deoxy PGE_1, butaprost and AH 13205 to cause relaxation with EECs (PGE_2 = 1) of 2, 42 and 6600 respectively. With this in mind, the effect of

the EP_4 receptor antagonist AH 23848B was examined. AH 23848B (10 μM) did not block the inhibitory response to PGE_2, but surprisingly, a potentiation of the effects of the lower concentrations of PGE_2 (0.001–0.1 μM) was observed. In addition, AH 23848B was also found to potentiate the weak inhibitory action of butaprost. The action of AH 23848B on butaprost first led us to believe that AH 23848B may have been acting as a partial agonist and not as an antagonist as described by Coleman *et al.* (1994), but this is unlikely as AH 23848B showed no inhibitory effect when incubated with neutrophils on its own. It seems unlikely that AH 23848B is acting as a phosphodiesterase inhibitor as potentiation of inhibition was still observed when PGE_2 was examined in IBMX treated neutrophils. Furthermore, this effect appears to be unrelated to the EP_4 receptor blocking activity of AH 23848B as potentiation of the adenosine-mimetic, NECA was also observed. Since AH 23848B was originally synthesized as a thromboxane receptor antagonist (Coleman *et al.*, 1990), possible interactions with the TP receptor are under investigation.

The EP_1/DP-receptor antagonist AH 6809 (Keery & Lumley, 1988), was found to produce a significant inhibition of the effects of 1 and 10 μM PGE_2 ($pA_2 = 6.04$), which is unlikely to be due to an effect through EP_1-receptors as both sulprostone and 17-phenyl PGE_2 were such weak inhibitors of superoxide generation. This may relate to a nonspecific effect of this compound, as AH 6809 was also found to inhibit the effects of the adenosine agonist NECA ($pA_2 = 6.83$) suggesting that AH 6809 is not acting on either EP or DP receptors.

Prostaglandins of the E series are known to activate membrane-bound adenylate cyclase in human neutrophils, and to increase intracellular cyclic AMP levels (Bourne *et al.*, 1971). It is possible therefore, that the increase in cyclic AMP induced by PGE_2 may in turn inhibit the release of O_2^- from human neutrophils. Indeed, many groups have studied the effects of PGE series prostaglandins on cyclic AMP in human neutrophils with relation to superoxide and lysosomal enzyme release (Zurier *et al.*, 1974). PGE_2 can be shown to activate membrane adenylate cyclase, but the increase in cyclic AMP in the whole cell is small, and can only be seen in the presence of a phosphodiesterase inhibitor to prevent cyclic AMP breakdown (Lad *et al.*, 1984). Although it has been proposed that these small increases in cyclic AMP are sufficient to inhibit various specific functions in the neutrophil (Lad *et al.*, 1984), the fact that these observations could be seen only in the presence of a phosphodiesterase inhibitor, and that cyclic AMP levels slowly increased over a 10 min time scale still cast doubt on the hypothesis that cyclic AMP is the second messenger mediating inhibition of O_2^- generation by PGE_2.

The demonstration that prostaglandins can elicit an increase in cyclic AMP is not on its own sufficient proof that cyclic AMP is the second messenger involved. The present study set about trying to accommodate three more criteria thought to be essential for concluding that cyclic AMP is the second messenger:-

First, we have shown that the adenylate cyclase inhibitor, SQ 22,536 (Harris *et al.*, 1979), significantly antagonized the inhibition of O_2^- generation produced by PGE_2. This result suggests strongly a causal link between cyclic AMP and PGE_2, as it shows that inhibition of the enzyme responsible for cyclic AMP production (adenylate cyclase) significantly reduces the action of PGE_2 on O_2^- generation. Unfortunately SQ 22,536 is not a very effective inhibitor of adenylate cyclase, and it is not possible to block adenylate cyclase activity completely.

Secondly, the inhibitory effects of PGE_2 are significantly potentiated after phosphodiesterase block with IBMX and rolipram. The inhibition of phosphodiesterase and the resultant increase in neutrophil cyclic AMP levels was itself found to inhibit the FMLP response by about 50% with both IBMX (0.25 mM) and high concentrations of rolipram. This demonstrates that the production of O_2^- by FMLP is susceptible to inhibition by increased cyclic AMP levels. In addition, the finding that lower concentrations of rolipram, which do not inhibit the FMLP response, are still capable of potentiating inhibition by PGE_2 suggests that this action results from the phosphodiesterase block and not the reduction in FMLP stimulus.

Further evidence that the effect of IBMX is unlikely to result from adenosine receptor block, is provided by the observation that neither ADA nor 8-PT shift the concentration-effect curve for PGE_2 in a similar manner to IBMX.

Thirdly, there is an impressive correlation ($r = 0.92$) between the increase in cyclic AMP levels (Armstrong & Talpain, 1994) and the inhibition of O_2^- generation particularly when the phosphodiesterase inhibitor (IBMX) is used to evaluate the inhibition of the superoxide generation, as is the case when cyclic AMP levels are measured. As would be predicted, a threshold increase in cyclic AMP is required before any inhibitory effect can be detected.

However, results with 8 bromo cyclic AMP were poor, but inhibition of neutrophil activation has previously been shown by Wong *et al.* (1980) using the cyclic AMP analogue dibutyryl adenosine 3',5'-monophosphate.

Taken together, these results suggest that cyclic AMP acts as a second messenger to mediate the inhibition by PGE_2 and EP_2 agonists of FMLP-induced superoxide formation. This has also been suggested by Li *et al.* (1993). The exact mechanism by which cyclic AMP inhibits O_2^- generation still remains to be elucidated. However, it has been demonstrated (Hecker *et al.*, 1989; Ney & Schror, 1991) that the increase in intracellular cyclic AMP produced by PGE_2 in human neutrophils is paralleled by a decrease in extracellular Ca^{2+} influx which may lead to the attenuation of receptor-mediated neutrophil activation by FMLP. It is interesting to note that Sedgwick *et al.* (1985) found that although cyclic AMP levels increased in all neutrophils stimulated by the three different neutrophil activators, FMLP, phorbol myristate acetate and serum-treated zymosan, inhibition of O_2^- was seen only in the neutrophils treated with FMLP. This finding suggests that the influence of prostaglandins on O_2^- production depends on the nature of the neutrophil activation stimulus, and that an increase in cyclic AMP in the activated neutrophil alone is not always sufficient to limit O_2^- generation. As a result of this, any conclusions drawn in this study must be restricted to FMLP.

In conclusion, the results of the present study suggest that inhibition of FMLP-induced superoxide generation by prostanoids is mediated by the occupation of EP_2 receptors and activation of adenylate cyclase, leading to the elevation of intracellular levels of cyclic AMP.

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