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# Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which $PGE_2$ inhibits the release of GM-CSF

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**1** The prostanoid receptor(s) on human airways smooth muscle (HASM) cells that mediates the inhibitory effect of $PGE_2$ on interleukin (IL)-1$\beta$-induced granulocyte/macrophage colony-stimulating factor (GM-CSF) release has been classified.

**2** IL-1$\beta$ evoked the release of GM-CSF from HASM cells, which was suppressed by $PGE_2$, 16,16-dimethyl $PGE_2$ (nonselective), misoprostol ($EP_2/EP_3$-selective), ONO-AE1-259 and butaprost (both $EP_2$-selective) with $pIC_{50}$ values of 8.61, 7.13, 5.64, 8.79 and 5.43, respectively. EP-receptor agonists that have selectivity for the $EP_1$- (17-phenyl-$\omega$-trinor $PGE_2$) and $EP_3$-receptor (sulprostone) subtypes as well as cicaprost (IP-selective), $PGD_2$, $PGF_{2\alpha}$ and U-46619 (TP-selective) were poorly active or inactive at concentrations up to 10 $\mu$M.

**3** AH 6809, a drug that can be used to selectively block $EP_2$-receptors in HASM cells, antagonised the inhibitory effect of $PGE_2$, 16,16-dimethyl $PGE_2$ and ONO-AE1-259 with apparent $pA_2$ values of 5.85, 6.09 and 6.1 respectively. In contrast, the $EP_4$-receptor antagonists, AH 23848B and L-161,982, failed to displace to the right the concentration–response curves that described the inhibition of GM-CSF release evoked by $PGE_2$ and ONO-AE1-259.

**4** Inhibition of GM-CSF release by $PGE_2$ and 8-Br-cAMP was abolished in cells infected with an adenovirus vector encoding an inhibitor protein of cAMP-dependent protein kinase (PKA) but not by H-89, a purported small molecule inhibitor of PKA.

**5** We conclude that prostanoid receptors of the $EP_2$-subtype mediate the inhibitory effect of $PGE_2$ on GM-CSF release from HASM cells by recruiting a PKA-dependent pathway. In addition, the data illustrate that caution should be exercised when using H-89 in studies designed to assess the role of PKA in biological processes.

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**Keywords:** Gene expression; prostanoid receptors; GM-CSF; ONO-AE1-259; $PGE_2$; human airways smooth muscle; $EP_2$-receptors; PKA inhibitor – cAMP-dependent protein kinase inhibitor (PKI$\alpha$); PKA inhibitor – H-89; Adenovirus vector – Ad5.CMV.PKI$\alpha$

**Abbreviations:** CTD, carboxy-terminal domain; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CSF, colony-stimulating factor; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; IL, interleukin.

## Introduction

Colony-stimulating factors (CSFs) such as granulocyte/macrophage colony-stimulating factor (GM-CSF) are responsible for the proliferation and differentiation of cells in the bone marrow (Metcalf, 1985; 1986). These cytokines also modulate the function of mature leukocytes, including eosinophils and neutrophils, promoting their activation and survival (Lopez *et al*., 1986; Giembycz & Lindsay, 1999). It is believed that by increasing the longevity of proinflammatory cells in tissues, CSFs may perpetuate airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) where eosinophils and neutrophils play a pathogenic role. It is now appreciated that human airways smooth muscle (HASM) cells have a substantial synthetic capacity and can contribute to inflammatory processes through the generation of a plethora of mediators including cytokines, chemokines and bioactive lipids (Johnson & Knox, 1997). Of relevance to the present study is the finding that HASM cells generate GM-CSF (Saunders *et al*., 1997; Lazzeri *et al*., 2001) that can be suppressed by endogenously synthesised or exogenously applied prostaglandin (PG) $E_2$ (Clarke *et al*., 2001; Lazzeri *et al*., 2001). The possibility exists, therefore, that agonism of specific prostaglandin receptors on HASM cells could provide an effective means of suppressing CSF production in diseases

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such as asthma and COPD and reduce, respectively, the eosinophil and neutrophil burden in the airways.

Five main classes of G-protein-coupled receptor for the naturally occurring prostanoid agonists have been defined and given the prefix DP, EP, FP, IP and TP (Coleman *et al.*, 1994b; Breyer *et al.*, 2001; Tsuboi *et al.*, 2002) where the first letter refers to the natural ligand most selective for that receptor. Molecular biological techniques subsequently confirmed this pharmacological classification with the cloning and expression of cDNAs for representatives of the five prostanoid receptors in a number of species including humans (Coleman *et al.*, 1994b; Breyer *et al.*, 2001; Tsuboi *et al.*, 2002). The finding that $PGE_2$ potently inhibits the release of GM-CSF from HASM cells in response to IL (interleukin)-1$\beta$ (Clarke *et al.*, 2001; Lazzeri *et al.*, 2001) suggests that this response is mediated by one or more prostanoid receptors of the EP-subtype. Currently, pharmacological evidence and primary sequence information of partial and full-length cDNA clones indicates the presence of at least four EP-receptor variants denoted $EP_1$, $EP_2$, $EP_3$ and $EP_4$ (Coleman *et al.*, 1994b; Narumiya *et al.*, 1999; Breyer *et al.*, 2001). These receptors can couple to several effector molecules (Breyer *et al.*, 2001), thereby mediating a diverse array of biological responses (Narumiya *et al.*, 1999; Kobayashi & Narumiya, 2002; Tsuboi *et al.*, 2002) that can now be reasonably well defined with agonists and antagonists that can discriminate between EP-receptor subtypes.

The objective of the present study was to characterise the prostanoid receptor(s) through which $PGE_2$ inhibits GM-CSF release from IL-1$\beta$-stimulated HASM cells and probe the molecular basis of this effect. To this end, naturally occurring and synthetic prostanoid agonists and antagonists that have selectivity for the EP-receptor subtypes were used. In addition, the role of the cAMP/cAMP-dependent protein kinase (PKA) cascade was assessed using an adenovirus (Ad5) vector encoding the $\alpha$-isoform of PKA inhibitor protein (PKI$\alpha$), which is potent, extremely selective for PKA (Olsen & Uhler, 1991; Scarpetta & Uhler, 1993; Collins & Uhler, 1997) and devoid of the problems associated with many small molecule protein kinase inhibitors (Engh *et al.*, 1996; Davies *et al.*, 2000).

## Methods

### *Isolation of HASM cells*

Tracheal rings from either lung or heart and lung transplantation donors (7 female, 17 male, age range: 17–57 years; median age 36.5 years) were dissected under sterile conditions in Hanks' balanced salt solution (HBSS; in mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, $Na_2HPO_4 \cdot 7H_2O$ 0.4, $CaCl_2 \cdot 2H_2O$ 1.3, $NaHCO_3$ 4.2 and glucose 5.6) supplemented with penicillin (100 U ml$^{-1}$), streptomycin (100 $\mu$g ml$^{-1}$) and amphotericin B (2.5 $\mu$g ml$^{-1}$). The smooth muscle layer was dissected free of adherent connective tissue and cartilage, and the epithelium was removed using a rounded scalpel blade. The smooth muscle was incubated (30 min; 37°C; 5% $CO_2$/air) in HBSS containing BSA (10 mg ml$^{-1}$), collagenase (type XI, 1 mg ml$^{-1}$) and elastase (type I, 3.3 U ml$^{-1}$). After the removal of any remaining connective tissue, the smooth muscle was chopped finely and incubated for a further 150 min in the enzyme solution described above with the elastase concentration increased to 15 U/ml. Dissociated cells were centrifuged ($100 \times g$, 5 min, 4°C) and resuspended in Dulbecco's-modified Eagle's medium (DMEM) containing heat-inactivated foetal calf serum (FCS; 10% v v$^{-1}$), sodium pyruvate (1 mM), L-glutamate (2 mM), nonessential amino acids (1×) and the antimicrobial agents detailed above.

### *Primary culture of HASM cells*

The HASM cell suspension was placed in a tissue culture flask (75 cm$^2$) containing 6 ml supplemented DMEM and allowed to adhere (~12 h) at 37°C in 5% $CO_2$/air. The culture medium was replaced after 4–5 days (12 ml) and thereafter every 3–4 days. When the cells reached confluence (~10–14 days) and demonstrated a typical 'hill and valley' appearance and positive immunostaining for $\alpha$-actin (routinely >95%, Belvisi *et al.*, 1997), they were seeded onto either 96-well plates (Costar UK Ltd., High Wycombe) at an initial density of 2000 cells per well or six well plates (Costar) at an initial density of 20,000 cell per well for cytokine release and Western blotting experiments, respectively. At subconfluence, the cells were growth arrested by being placed in DMEM containing apotransferin (5 $\mu$g ml$^{-1}$), insulin (1 $\mu$M), ascorbate (100 $\mu$M) and BSA (0.1% w v$^{-1}$) for 24 h. The medium was replaced with DMEM containing 3% FCS v v$^{-1}$ and drugs or appropriate vehicles as described below.

### *Infection of HASM cells with Ad5.CMV.PKI$\alpha$*

In some experiments, subconfluent, growth-arrested HASM cells were infected (MOI = 100) with an $E1^-/E3^-$ replication-deficient Ad5 vector (Ad5.CMV.PKI$\alpha$) containing a 251 bp DNA fragment encoding the complete amino-acid sequence of PKI$\alpha$ (Day *et al.*, 1989; Meja *et al.*, 2004), downstream of the constitutively active CMV immediately early promoter (Gomez-Foix *et al.*, 1992; Lum *et al.*, 1999). After 48 h, cells were processed for GM-CSF release and Western blotting as described below. To control for possible biological effects of the virus *per se*, the vector, Ad5.CMV.Null, expressing no transgene, was used in parallel. Preliminary experiments using immunofluorescence microscopy established that >95% of cells expressed PKI$\alpha$ 48 h after infection. This was determined by enumerating the number of HASM cells expressing the transgene as a percentage of total number of cells that counterstained with the nuclear marker, 4′,6-diamidino-2-phenylindole. Ad5.CMV.PKI$\alpha$ and Ad5.CMV.Null at an MOI of 100 had no effect on HASM cell viability 48 h after infection (data not shown).

### *Measurement of GM-CSF*

HASM cells (naïve and virus-treated) were pretreated for 30 min with indomethacin (10 $\mu$M) and, where indicated, receptor antagonist before being exposed for a further 5 min or 30 min to prostanoid agonists or 8-Br-cAMP, respectively. IL-1$\beta$ (100 pg ml$^{-1}$) was then added and the cells were incubated at 37°C in a thermostatically controlled incubator under a 5% $CO_2$ atmosphere. At 24 h, the amount of GM-CSF released into the culture supernatant was quantified by a sandwich ELISA (human DuoSet® development system, R&D Systems Europe, Abingdon) according to the

manufacturer's instructions. The detection limit of this assay is 7.8 pg ml$^{-1}$.

*Western blot analysis*

HASM cells were treated with 3% FCS for 24 h. The medium was removed and the cells washed with HBSS, lysed and proteins extracted in 20 mM Tris HCl – pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% (v v$^{-1}$) Nonidet P-40, 0.05% (w v$^{-1}$) sodium deoxycholate, 0.025% (w v$^{-1}$) SDS and 0.1% (v v$^{-1}$) Triton X-100 supplemented with PMSF (0.1 mg ml$^{-1}$), leupeptin (10 $\mu$g ml$^{-1}$) and aprotinin (25 $\mu$g ml$^{-1}$). Insoluble protein was removed by centrifugation (10,000 $\times$ *g*; 3 min) and aliquots of the resulting supernatant were diluted 1 : 4 in Laemmeli buffer (62.5 mM Tris-HCl – pH 6.8, 10% (v v$^{-1}$) glycerol, 1% (w v$^{-1}$) SDS, 1% (v v$^{-1}$) $\beta$-mercaptoethanol, 0.01% (v v$^{-1}$) bromophenol blue) and boiled for 5 min. Denatured proteins (25 $\mu$g) were separated by SDS–PAGE using a 4–20% gradient gel (BioRad; Hemel Hempstead) and transferred to Hybond enhanced chemiluminescence (ECL) membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire) in Tris buffer (Tris base 50 mM – pH 8.3, glycine 192 mM, 20% v v$^{-1}$ methanol). The nitrocellulose was incubated overnight in Tris-buffered saline plus Tween-20 (TBS-T) (25 mM Tris-base – pH 7.4, 150 mM NaCl, 0.1% v v$^{-1}$ Tween 20) containing 5% (w v$^{-1}$) non-fat dry milk. The filters were incubated for 1 h at room temperature in TBS-T containing 5% non-fat dry milk and an anti-human PKI$\alpha$, pCREB or $\beta$-actin polyclonal antibody (diluted 1 : 500, 1 : 1000 and 1 : 2000, respectively) as indicated. Membranes were washed with TBS-T and incubated with horseradish peroxide-conjugated sheep, anti-rabbit IgG (diluted 1 : 4000) in TBS-T/5% nonfat dry milk for 1 h at room temperature. The nitrocellulose was washed again in TBS-T and developed using ECL™ western blotting detection reagents on Kodak X-OMAT-S film.

*Cell viability*

At the end of each experiment, cell viability was determined colorimetrically by measuring the reduction of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), to formazan by mitochondrial dehydrogenases (Mosmann, 1983; Hussain *et al*., 1993).

*Drugs, antibodies and analytical reagents*

IL-1$\beta$ was from R&D systems (Abingdon, Oxon, U.K.), DMEM and HBSS were from Flow Laboratories (Rickmansworth, Hertfordshire, U.K.) and nonessential amino acids were purchased from Life Technologies (Paisley, U.K.). Indomethacin, FCS, MTT, PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, U-46619 (9,11-dideoxy-9$\alpha$,11$\alpha$-methanoepoxy prostaglandin F$_{2\alpha}$) and H-89 were from Sigma-Aldrich (Poole, Dorset, U.K.). 16,16-Dimethyl PGE$_2$, 17-phenyl-$\omega$-trinor PGE$_2$, AH 6809 (6-isopropoxy-9-oxoxanthine-2-carboxylic acid), SQ 29,548 (*1S*-[1$\alpha$,2$\alpha$(Z),3$\alpha$,4$\alpha$]]-7-[3-[[2-[(phenylamino) carbonyl]hydrazino]methyl]-7-oxa bicyclo[2.2.1]hept-2-y1]-5-heptenoic acid), AH 23848B ([1$\alpha$(Z),2$\beta$,5$\alpha$]-($\pm$)-7-[5-[[(1,1′-biphenyl]-4-yl-methoxy)-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptanoic acid), sulprostone, misoprostol methyl ester and butaprost methyl ester were obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.). All other synthetic prostanoid reagents were donated by the following: cicaprost (Schering AG, Berlin, Germany); ONO-AE1-259 ((*16S*)-9-deoxy-9$\beta$-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro-PGE$_2$ – sodium salt; Ono Pharmaceuticals, Osaka, Japan) and L-161,982 ([4′-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-dihydro-[1,2,4]triazol-4-ylmethyl]-biphenyl-2-sulphonic acid (3-methyl-thiophene-2-carbonyl)-amide]; Merck Frosst, Montreal, Canada). Goat anti-human PKI$\alpha$ (code sc#1944) and goat anti-human $\beta$-actin (code sc# 1615) were from Autogen Bioclear (Calne, Wiltshire, U.K.). pCREB (code 9191S) was purchased from New England Biolabs (Hitchin, Hertfordshire, U.K.).

*Data and statistical analyses*

Data points, and values in the text and figure legends, represent the mean$\pm$s.e.m. of '*n*' independent determinations using tissue from different donors. The concentration–response curves were analysed by least-squares, nonlinear iterative regression with the 'PRISM' curve fitting program (GraphPad software, San Diego, U.S.A.) and pEC$_{50}$ and pIC$_{50}$ values were subsequently interpolated from curves of best-fit. Equieffective molar concentration ratios (e.c.r.) were calculated using the formula: IC$_{50}$ PGE analogue/IC$_{50}$ PGE$_2$.

Antagonist affinity was calculated using the equation $pK_B = \log(\text{CR-1}) - \log[B]$ (Schild, 1949), where CR is the concentration ratio calculated from the EC$_{50}$ of agonist in the presence of the antagonist divided by the EC$_{50}$ of the agonist alone, $K_B$ is the equilibrium dissociation constant and $[B]$ is the concentration of antagonist. In the experiments described herein, the term apparent p$A_2$ is substituted for p$K_B$ as antagonists were used at one concentration only, which precludes assumptions being made about the nature of the antagonism.

Where appropriate, data were analysed statistically using Student's paired *t*-test or by one-way ANOVA/Newman–Keuls multiple comparison test. The null hypothesis was rejected when $P < 0.05$.

## Results

We have reported previously that IL-1$\beta$ promotes a time- and concentration-dependent release of GM-CSF from HASM cells with a $t_{1/2}$ and EC$_{50}$ of >18 h and 16 pg ml$^{-1}$, respectively (Clarke *et al*., 2001). In the experiments described herein, IL-1$\beta$ was used at 100 pg ml$^{-1}$ ($\sim$EC$_{90}$) and GM-CSF was measured in the culture supernatant 24 h after addition of the stimulus. None of the compounds or their vehicles used in these experiments affected cell viability as determined by the reduction of MTT to formazan. None of the vehicles used had any significant effect on GM-CSF release (data not shown).

*Effect of naturally occurring prostaglandins, cicaprost and U-46619 on GM-CSF release*

PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, U-46619 and cicaprost had no effect on GM-CSF release from indomethacin (10 $\mu$M; 30 min)-pretreated HASM cells. However, the GM-CSF elaborated in response to IL-1$\beta$ was suppressed by PGE$_2$ in a concentration-dependent manner with a maximal effect and pIC$_{50}$ of 79% and 8.61, respectively (Figure 1, Table 1). Cicaprost was also

active but the concentration–response curves that described the suppression of GM-CSF were complex and better described by a two-site (mean $r^2 = 0.956$) rather than single sigmoidal function (mean $r^2 = 0.765$; Figure 1). Analysing the data in this way yielded pIC$_{50}$ values for the high- and low-affinity components of 9.45, which accounted for 34.1±5.5% of the effect, and 4.69 (estimate), respectively. PGD$_2$, PGF$_{2\alpha}$ and U-46619 had no significant effect on GM-CSF release (Figure 1, Table 1).

### *Effect of EP-selective prostanoid agonists on GM-CSF release*

To gain information on the possible role of an EP-receptor subtype in mediating the inhibitory effect of PGE$_2$ on GM-CSF release, a variety of PGE analogues, which differ in their selectivity for the EP$_1$-, EP$_2$-, EP$_3$- and EP$_4$-receptor subtypes, were examined (Figure 2, Table 1). By themselves none of the ligands had any effect on GM-CSF release. However, in IL-1$\beta$-stimulated HASM cells, 16,16-dimethyl PGE$_2$, ONO-AE1-259, misoprostol and butaprost suppressed the release of GM-CSF in a concentration-dependent manner, with a maximum inhibition of 83, 68, 65 and 56%, respectively, at the highest concentration (10 $\mu$M) of drug examined (Figure 2). The rank order of agonist potency was ONO-AE1-259 > 16,16-dimethyl PGE$_2$ > misoprostol > butaprost. 17-Phenyl-$\omega$-trinor PGE$_2$ suppressed GM-CSF output at concentrations above 1 $\mu$M

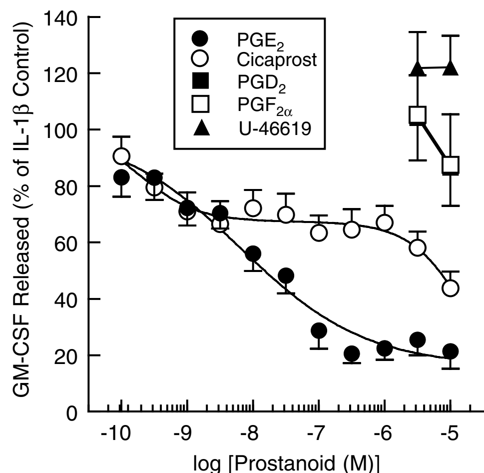


**Figure 1** Effect of prostanoids on IL-1$\beta$-induced GM-CSF release. Adherent HASM cells were pretreated for 5 min with varying concentrations of PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, cicaprost and U-46619 before being exposed to IL-1$\beta$ (100 pg ml$^{-1}$). Cells were maintained at 37°C in a thermostatically controlled incubator under a 5% CO$_2$ atmosphere and the amount of GM-CSF released into the culture supernatant was quantified at 24 h by a sandwich ELISA. Each data point represents the mean±s.e.m. of four to 13 determinations (see Table 1) using tissue from different donors. Indomethacin (10 $\mu$M) was present through the experiment. Note that the data for PGD$_2$ and PGF$_{2\alpha}$ are superimposed. See Methods for further details.

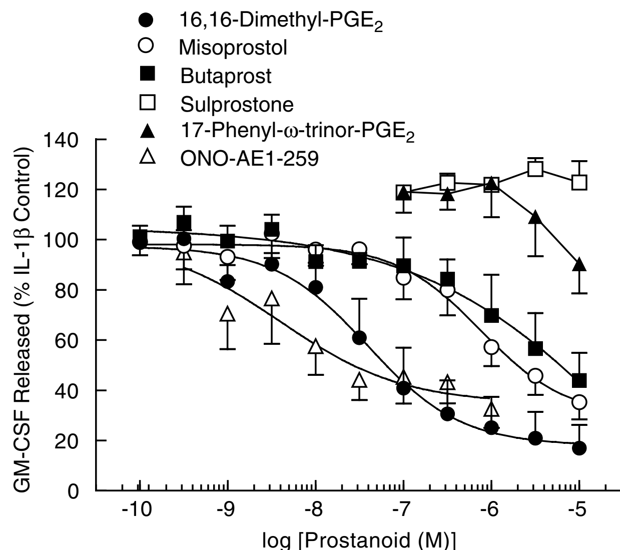


**Figure 2** Effect of EP-selective prostanoid agonists on IL-1$\beta$-induced GM-CSF release. Adherent HASM cells were pretreated for 5 min with varying concentrations of six synthetic PGE analogues before being exposed to IL-1$\beta$ (100 pg ml$^{-1}$). Cells were maintained at 37°C in a thermostatically controlled incubator under a 5% CO$_2$ atmosphere and the amount of GM-CSF released into the culture supernatant was quantified at 24 h by a sandwich ELISA. Each data point represents the mean±s.e.m. of four to 13 determinations (see Table 1) using tissue from different donors. Indomethacin (10 $\mu$M) was present through the experiment. See Methods for further details.

**Table 1** Potency of prostanoid and EP/IP-selective agonists at suppressing GM-CSF generation

| *Prostanoid* | n | *Receptor selectivity*[a] | *Inhibition of GM-CSF release pIC$_{50}$* | *e.c.r. PGE$_2$ = 1* |
|---|---|---|---|---|
| PGE$_2$ | 13 | EP$_1$≈EP$_2$ ≈EP$_3$≈EP$_4$ | 8.61±0.43 (78.6±6.2) | 1 |
| ONO-AE1-259 | 8 | EP$_2$>>>EP$_1$=EP$_3$=EP$_4$ | 8.79±0.26 (8.79±0.3) | 0.7 |
| 16,16-dimethyl PGE$_2$ | 7 | EP$_2$⩾EP$_3$=EP$_1$>EP$_4$ | 7.13±0.37 (83.0±9.2) | 30 |
| Misoprostol | 5 | EP$_2$=EP$_3$>EP$_1$>EP$_4$ | 5.64±0.25 (64.7±6.9) | 935 |
| Butaprost | 4 | EP$_2$>>EP$_1$>EP$_3$>EP$_4$ | 5.43±0.26 (56.1±10.9) | 1518 |
| Cicaprost[b] | 11 | IP>EP$_4$ | 9.45±0.26 | 0.14 |
| | | | >5 | >4081 |
| PGF$_{2\alpha}$ | 4 | FP | >5 (12.5±17.9%) | >4081 |
| PGD$_2$ | 4 | DP | >5 (13.4±13.6%) | >4081 |
| U-46619 | 9 | TP | >5 (−22.1±11.2%) | >4081 |
| 17-phenyl-$\omega$-trinor PGE$_2$ | 4 | EP$_1$>EP$_3$>EP$_2$>EP$_4$ | >5 (9.7±11.8%) | >4081 |
| Sulprostone | 4 | EP$_3$>EP$_1$>>EP$_2$>EP$_4$ | >5 (−22.7±8.6%) | >4081 |

Values in parentheses show the percentage inhibition of GM-CSF release evoked by 10 $\mu$M prostanoid agonist. Equieffective concentration ratios >1 and <1 indicate that the agonist in question is less and more potent than PGE$_2$, respectively.
[a]Selectivity derived from studies in isolated cells and tissues.
[b]Concentration–response curve described by a two site sigmoidal function.

whereas sulprostone was inactive at all concentrations examined (Figure 2, Table 1).

*Effect of an $EP_2$-receptor antagonist on the inhibition of GM-CSF release evoked by $PGE_2$, 16,16-dimethyl $PGE_2$ and ONO-AE1-259*

AH 6809 is an antagonist at the human $EP_1$-, $EP_2$- and DP-receptor subtypes (Coleman *et al.*, 1987; Keery & Lumley, 1988). As 17-phenyl-$\omega$-trinor $PGE_2$ ($EP_1$-selective) and $PGD_2$ were inactive in this system (Table 1, Figure 2), AH 6809 was used to determine if $EP_2$ receptors mediated the inhibitory effects of $PGE_2$, 16,16-dimethyl $PGE_2$ and ONO-AE1-259 on GM-CSF release. Pretreatment of HASM cells with AH 6809 (10 $\mu$M) produced a rightwards shift of the concentration–response curve that described the inhibition of GM-CSF release by $PGE_2$, 16,16-dimethyl $PGE_2$ and ONO-AE1-259 from which apparent p$A_2$ values of $5.85 \pm 0.31$, $6.09 \pm 0.22$ and $6.1 \pm 0.7$ were derived, respectively (Figure 3). These affinity estimates were not statistically significant from one another ($P > 0.05$; one-way ANOVA).

*Effect of $EP_4$-receptor antagonists on the inhibition of GM-CSF release evoked by $PGE_2$ and ONO-AE1-259*

To evaluate the role of $EP_4$-receptors in mediating suppression of GM-CSF release, the antagonists AH 23848B (Coleman *et al.*, 1994a) and L-161,982 (Machwate *et al.*, 2001) were employed using $PGE_2$ and ONO-AE1-259 as agonists. In the presence of AH 23848B or L-161,982, at concentrations $\sim$10 and $\sim$100 times higher than their affinity at $EP_4$-receptors, respectively, the inhibitory effect of $PGE_2$ and ONO-AE1-259 was not significantly altered in terms of $IC_{50}$ or maximal inhibitory response (Figure 4).

*Effect of 8-Br-cAMP on IL-1$\beta$-induced GM-CSF release*

Pretreatment of HASM cells with 8-Br-cAMP inhibited the release of GM-CSF from IL-1$\beta$ (100 pg/ml)-stimulated HASM cells with a $pIC_{50}$ of $3.76 \pm 0.14$ ($n = 3$). At the highest concentration of drug tested (1 mM), the elaboration of GM-CSF was suppressed by $96.2 \pm 3.1\%$ ($n = 3$).

*Role of PKA in the action of $PGE_2$ on GM-CSF release*

To determine the role of PKA in mediating the effect of $PGE_2$ on GM-CSF release from HASM cells H-89, a purported selective inhibitor of PKA (Chijiwa *et al.*, 1990), and a virus vector, Ad5.CMV.PKI$\alpha$ (Lum *et al.*, 1999; Meja *et al.*, 2004), containing DNA encoding the complete amino-acid sequence of PKI$\alpha$, an endogenous, potent and highly selective inhibitor of PKA (Glass *et al.*, 1986; Olsen & Uhler, 1991) were employed. In the virus studies, western blotting was used to confirm expression of the PKI$\alpha$ transgene. In uninfected cells, PKI$\alpha$ was not detected in any experiment. However, 48 h after infection with Ad5.CMV.PKI$\alpha$ (MOI = 100), a single peptide was labelled by the anti-PKI$\alpha$ antibody that migrated as a 12 kDa band on SDS polyacrylamide gels (Figure 5). In preliminary studies, the efficiency of transgene expression at 48 h was found to be >95% (see Methods for details).

HASM cells exposed to IL-1$\beta$ elaborated GM-CSF in an amount that was not significantly altered by H-89 (10 $\mu$M; 30 min) or following infection with Ad5.CMV.PKI$\alpha$ or Ad5.CMV.Null (MOI = 100; 48 h) (Figure 6a,b). $PGE_2$ (10 $\mu$M) and 8-Br-cAMP (1 mM), which was used as a positive control, suppressed IL-1$\beta$-stimulated GM-CSF release by a mechanism that was prevented in cells expressing the PKI$\alpha$ transgene but *not* those infected with the empty virus or pretreated with H-89 (Figure 6a, b). Paradoxically, the phosphorylation by 8-Br-cAMP and $PGE_2$ of CREB, a well established substrate for PKA, was abolished in HASM cells

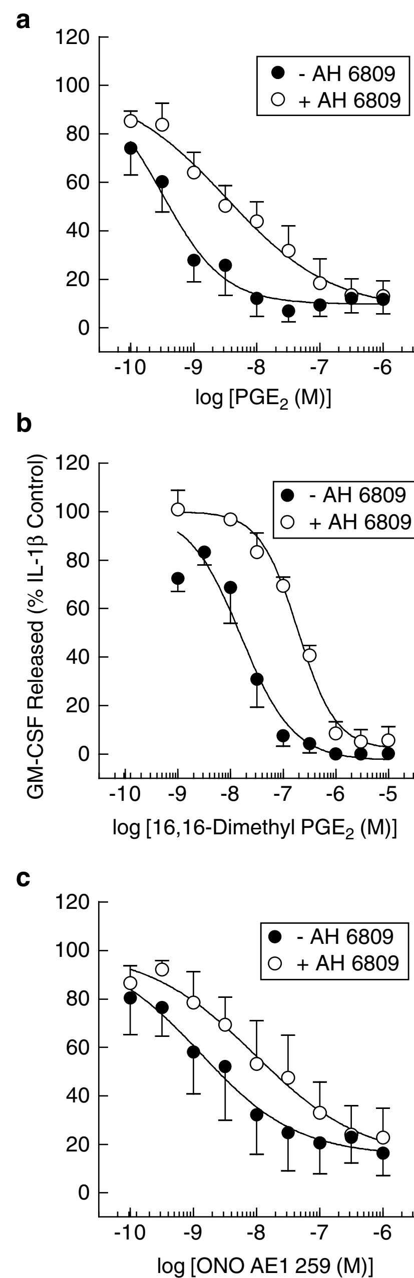


**Figure 3** Effect of AH 6809 on the inhibition of IL-1$\beta$-induced GM-CSF evoked by $PGE_2$, 16,16-dimethyl $PGE_2$ and ONO-AE1-259. Adherent HASM cells were pretreated (30 min) concurrently with indomethacin and AH 6809 (both 10 $\mu$M) and then for a further 5 min with $PGE_2$ (a), 16,16-dimethyl (b) or ONO-AE1-259 (c). IL-1$\beta$ (100 pg ml$^{-1}$) was then added to the cells and the GM-CSF released into the culture medium was quantified at 24 h by a sandwich ELISA. Each data point represents the mean $\pm$ s.e.m. of three to five determinations using tissue from different donors.

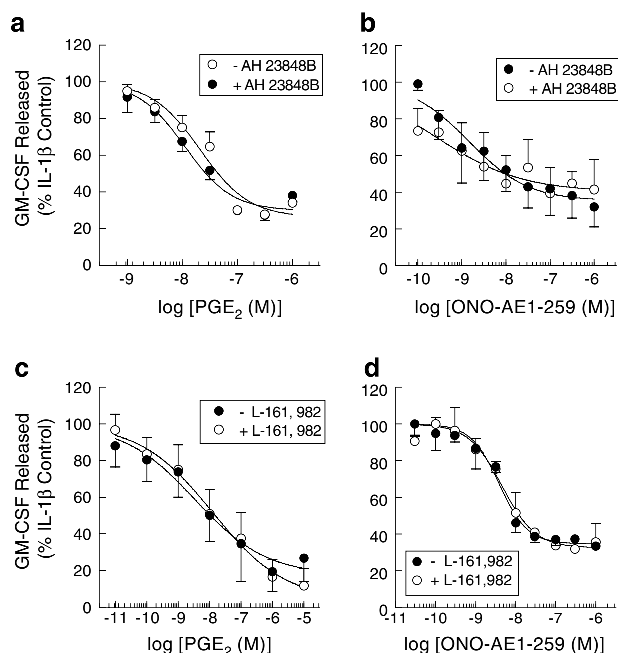


**Figure 4** Effect of AH 23848B and L-161,982 on the inhibition of IL-1$\beta$-induced GM-CSF release evoked by $PGE_2$ and ONO-AE1-259. Adherent HASM cells were pretreated (30 min) concurrently with indomethacin (10 $\mu$M) and either AH 23848B (30 $\mu$M) or L-161,982 (2 $\mu$M) and then for a further 5 min with $PGE_2$ (a and b) or ONO-AE1-259 (c and d). IL-1$\beta$ (100 pg ml$^{-1}$) was then added to the cells and the GM-CSF released in to the culture medium was quantified at 24 h by a sandwich ELISA. Each data point represents the mean $\pm$ s.e.m. of three to five determinations using tissue from different donors.

infected with Ad5.CMV.PKI$\alpha$ *and* H-89 confirming that PKA was inhibited by both interventions (Figure 6c).

## Discussion

We have reported previously that endogenously synthesised and exogenously applied $PGE_2$ can negatively regulate the elaboration of GM-CSF from HASM cells elicited by the proinflammatory cytokine, IL-1$\beta$ (Clarke *et al.*, 2001; Lazzeri

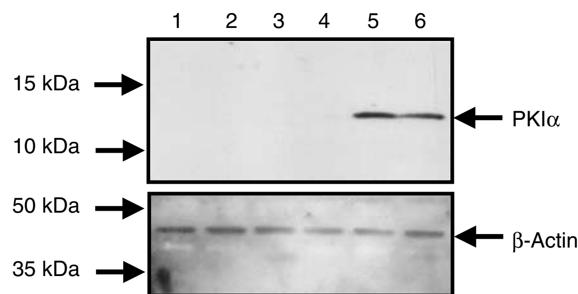


**Figure 5** Expression of PKI$\alpha$ in HASM cells infected with Ad5.CMV.PKI$\alpha$. Adherent cells were cultured until 50% confluent and then infected with Ad5.CMV.Null, Ad5.CMV.PKI$\alpha$ (MOI = 100) or left untreated (naïve) for 48 h at 37°C. Cells were growth arrested in serum-free medium and processed by western blotting for PKI$\alpha$ expression (a) and the house-keeping protein, $\beta$-actin (b). Data are representative of three independent determinations using tissue from different donors. See Methods for further details. (Key: lanes 1 & 2, Naïve; lanes 3 & 4, Ad5.CMV.Null; lanes 5 & 6, Ad5.CMV.PKI$\alpha$).

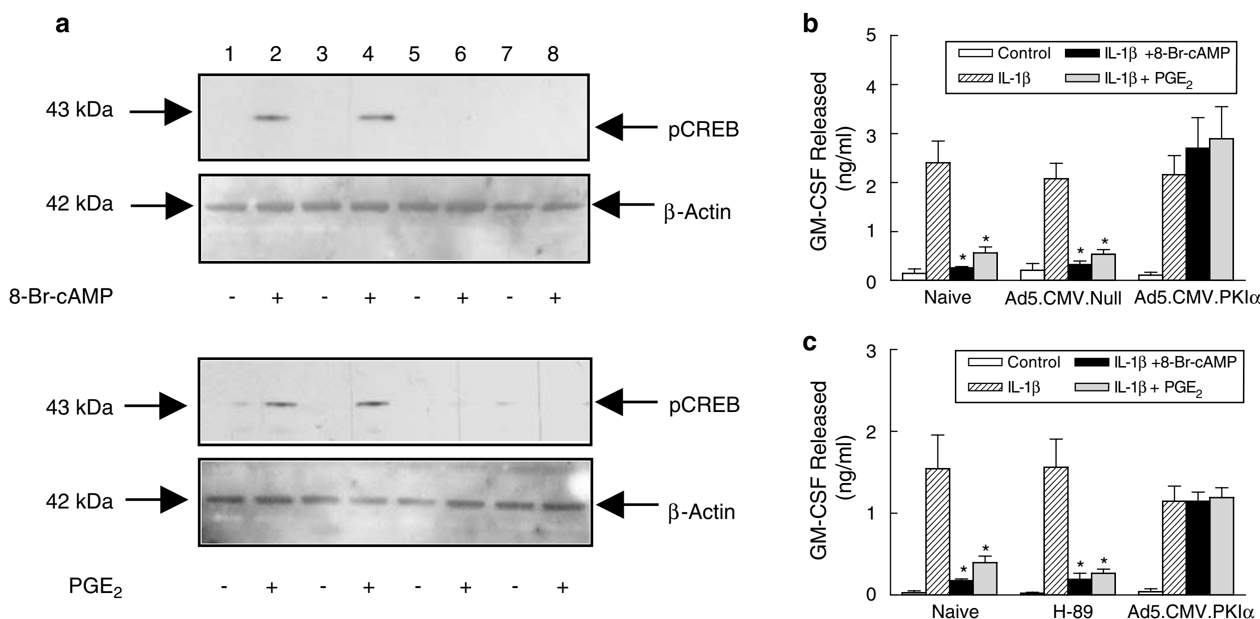


**Figure 6** Effect of H-89 and PKI$\alpha$ on 8-Br-cAMP- and $PGE_2$-induced GM-CSF release from and CREB phosphorylation in HASM cells. Adherent cells were pretreated with H-89 (10 $\mu$M; 30 min) or infected with Ad5.CMV.Null or Ad5.CMV.PKI$\alpha$ (MOI = 100; 48 h). 8-Br-cAMP (1 mM) or $PGE_2$ (10 $\mu$M) were then added for 30 min. At this point, cells were either processed immediately for CREB phosphorylation by western blotting (a) or exposed to IL-1$\beta$ (100 pg ml$^{-1}$) for 24 h to promote GM-CSF release, which was measured by ELISA (b and c). Each bar represents the mean $\pm$ s.e.m. of four independent determinations using tissue from different donors. Indomethacin (10 $\mu$M) was present throughout the experiment. See Methods for further details. *$P < 0.05$, significant inhibition of IL-1$\beta$-stimulated GM-CSF release. (Key: lanes 1 & 2, Naïve; lanes 3 & 4, Ad5.CMV.Null; lanes 5 & 6, Ad5.CMV.PKI$\alpha$, lanes 7 & 8, H-89).

et al., 2001). The experiments described in the present study were designed to further this work by characterising the prostanoid receptor(s) and second messenger pathway through which PGE$_2$ mediates this inhibitory effect. As PGE$_2$ was the most potent prostanoid at suppressing GM-CSF release from HASM cells, we reasoned that this might reflect the activation of an EP-receptor subtype. This conclusion was supported by the additional findings that cicaprost, an IP-receptor agonist, was a weak inhibitor of GM-CSF release and U-46619 (TP-agonist), PGD$_2$ and PGF$_{2\alpha}$ were inactive.

To identify the inhibitory EP-receptor(s) on HASM cells, a pharmacological analysis was performed by examining a panel of agonists and antagonists that have varying degrees of selectivity for the EP-receptor subtypes. The finding that 17-phenyl-$\omega$-trinor PGE$_2$ was greater than three orders of magnitude less active than PGE$_2$ in this system provides persuasive evidence that the elaboration of GM-CSF from HASM cells is not mediated by receptors of the EP$_1$-subtype (Lawrence *et al*., 1992).

EP$_2$- and EP$_4$-receptors can couple to adenylyl cyclase via Gs and enhance cAMP biosynthesis (Coleman *et al*., 1994a; Breyer *et al*., 2001). As 8-Br-cAMP was found to suppress GM-CSF release from HASM cells, it was considered likely that the EP$_2$- and/or EP$_4$-receptor might mediate the inhibitory effect of PGE$_2$ and related ligands in this system. Although agonism of certain splice variants of the human EP$_3$-receptor (e.g. EP$_{3B}$, EP$_{3C}$) can also couple positively to adenylyl cyclase (Namba *et al*., 1993), the failure of sulprostone to suppress GM-CSF release suggests that the EP$_3$-subtype does not regulate the expression of the *CSF2* gene in HASM cells. As illustrated in Figure 3, PGE$_2$, 16,16-dimethyl PGE$_2$, butaprost, misoprostol and ONO-AE1-259 effectively reduced the elaboration of GM-CSF. Evidence that these ligands were acting through EP$_2$-receptors was derived primarily from two pieces of data. First, the agonist activity of butaprost and ONO-AE1-259 reported in the present study is considered diagnostic of EP$_2$-receptors (Boie *et al*., 1997; Kiriyama *et al*., 1997; Suzawa *et al*., 2000). Second, AH 6809 antagonised the inhibitory effect of PGE$_2$ and related ligands with an affinity consistent with EP$_2$-receptor-mediated events in other tissues including human myometrium (p$A_2 = 5.85$, (Hillock & Crankshaw, 1999) and the human recombinant EP$_2$-subtype expressed in Chinese hamster ovary (CHO) cells (p$A_2 = 6.5$ (Woodward *et al*., 1995). It is noteworthy that AH 6809 is also an antagonist at the EP$_1$- and DP-receptor (Coleman *et al*., 1987; Keery & Lumley, 1988; Woodward *et al*., 1995). However, as neither 17-phenyl-$\omega$-trinor PGE$_2$ nor PGD$_2$ inhibited GM-CSF release, AH 6809 was used as a selective antagonist at the human EP$_2$-receptor. Despite antagonist affinity estimates implicating EP$_2$-receptors in mediating the inhibition of GM-CSF release, several observations were not entirely consistent with this conclusion. For example, a prediction based on data obtained with established EP$_2$-receptor-containing tissues such as the rabbit ear artery and cat trachea is that butaprost should be 10- to 100-times less potent than PGE$_2$ (Gardiner, 1986; Coleman *et al*., 1988; Nials *et al*., 1993; Lydford *et al*., 1996). However, in HASM cells, butaprost was considerably weaker than predicted with an e.c.r. of 1518 relative to PGE$_2$. Similarly, while misoprostol effectively reduced GM-CSF release, it was 935-times less potent than PGE$_2$ unlike its activity on the rabbit ear artery and the cat trachea where it is equieffective with the natural ligand (Coleman *et al*., 1988; Lydford *et al*., 1996). Consequently, the rank order of agonist potency for the suppression of GM-CSF output from HASM cells was markedly different from other EP$_2$-receptor-containing tissues. However, rather than invoke the involvement of multiple or a novel EP-receptor subtype(s), we suggest that a likely cause of these discrepancies is our use of the methyl esters of butaprost and misoprostol, which are considerably less potent (typically 10–300-times) than their respective free acids (Tsai *et al*., 1991; Abramovitz *et al*., 2000; Tani *et al*., 2001). For example, butaprost free acid has an affinity ($K_i \sim 80$ nM) for the human and murine recombinant EP$_2$-receptor subtype that is 39- and 32-fold higher than butaprost methyl ester ($K_i \sim 2.9\,\mu$M) respectively (Abramovitz *et al*., 2000; Tani *et al*., 2001). A more dramatic difference is seen with misoprostol where the free acid is $>300$-times more potent ($K_i = 34$ nM) than the methyl ester ($K_i = 10.3\,\mu$M) (Abramovitz *et al*., 2000). If this interpretation is correct, then the present study highlights a fundamental difference between HASM cells and other EP$_2$-receptor-expressing preparations where the methyl esters of misoprostol and butaprost have the expected e.c.r. relative to PGE$_2$. Mechanistically, this would imply that unlike many tissues HASM cells lack appreciable esterase activity and cannot convert esterified compounds such as misoprostol and butaprost to their free acids. Thus, these apparent anomalies not withstanding, the data obtained in the present study provide good evidence that PGE$_2$ suppresses GM-CSF release by interacting with prostanoid receptors of the EP$_2$-subtype. This conclusion is consistent with the expression by cultured HASM cells of mRNA and protein for the EP$_2$-receptor subtype (Clarke, D.L., Belvisi, M.G., Smith, S.J., Yacoub, M.H., Meja, K.K., Newton, R., Slater, D.M., Giembycz, M.A. – manuscript under review).

In other human tissues including synovial fibroblasts (Inoue *et al*., 2002), monocyte-derived macrophages (Takayama *et al*., 2002) and peripheral blood mononuclear cells (Takahashi *et al*., 2002), PGE$_2$ has been shown to regulate cytokine, chemokine and adhesion molecule expression by acting through EP$_4$-receptors. The failure of two EP$_4$-receptor antagonists, AH 23848B and L-161,982 (Coleman *et al*., 1994a; Machwate *et al*., 2001), to displace to the right the concentration-response curves that described the inhibition of GM-CSF release by PGE$_2$ and ONO-AE1-259 at a concentration $\sim 10$ and 100 times greater than their affinity at EP$_4$-receptors, respectively, indicates that this is not the case in HASM cells. This conclusion is further supported by the finding that PGE$_2$ did not suppress the elaboration of GM-CSF in the subnanomolar concentration range, which is typical of many other EP$_4$-receptor-expressing preparations (Coleman *et al*., 1994b).

Agonism of EP$_2$-receptors evokes responses that are thought to rely exclusively on the activation of the cAMP/PKA cascade (Tsuboi *et al*., 2002). However, in many biological systems, compelling evidence implicating this signalling pathway is difficult to gain as many compounds marketed as PKA inhibitors, such as H-89, are isoquinolinesulphonyl derivatives, which are remarkably nonselective (Davies *et al*., 2000), presumably because they block a conserved ATP-binding site found among many protein kinases (Engh *et al*., 1996). The reported limitations in H-89 prompted us to adopt an additional approach to establish the role of PKA in the inhibition, by PGE$_2$ and 8-Br-cAMP, of IL-1$\beta$-induced GM-CSF release. Thus, HASM cells were infected with an

adenovirus vector encoding the complete amino-acid sequence of PKIα, one of three endogenous, potent ($K_i \sim 50$–100 pM) and highly selective inhibitors of PKA (Olsen & Uhler, 1991; Scarpetta & Uhler, 1993; Collins & Uhler, 1997; Meja *et al*., 2004). Indeed, PKIα, at a concentration $\sim 10^6$ times higher than its affinity for PKA, does not inhibit the highly homologous cGMP-dependent protein kinase (Glass *et al*., 1986) to which it is most closely related (Takio *et al*., 1984). As shown in Figure 6, CREB phosphorylation and the inhibition of GM-CSF release evoked by PGE$_2$ and 8-Br-cAMP was abolished in HASM cells expressing PKIα, indicating that the *CSF2* gene is negatively regulated, directly or indirectly, by PKA. H-89 also prevented PGE$_2$- and 8-Br-cAMP-induced CREB phosphorylation under identical experimental conditions but, in contrast to PKIα-expressing cells, failed to block the reduction in GM-CSF output. These results agree with data published by Takayama *et al*. (2002), who found that H-89 abolished CREB phosphorylation in human monocyte-derived macrophages evoked by PGE$_2$ and 8-Br-cAMP but not the inhibition of MIP-1$\beta$ release. Similarly, the ability of 8-Br-cAMP and $\beta_2$-adrenoceptor agonists to suppress the release of eotaxin from TNFα-stimulated HASM cells was not prevented by H-89 (Pang & Knox, 2001). Taken together, these results imply that although H-89 abolishes cAMP-dependent CREB phosphorylation, other signalling intermediates are affected that oppose the functional consequences associated with the inhibition of PKA. The identity of these additional intracellular targets in HASM cells is unknown and was not a subject of this investigation. However, isoquinoline sulphonamides can bind many proteins with high affinity including certain carboxy-terminal domain kinases, which have a profound influence on gene transcription (Dubois *et al*., 1994). Based on these data, we suggest that caution should be exercised when using H-89 in studies designed to assess the role of PKA in biological responses.

Cicaprost (Sturzebecher *et al*., 1986) also suppressed GM-CSF release from HASM cells although the concentration–response relationship was complex and better accommodated by a two-site sigmoidal function. The IC$_{50}$ of cicaprost for the high-affinity component, which accounted for $\sim 34\%$ of the response, was comparable to that found on well characterised IP-receptor containing tissues including human platelets and vascular smooth muscle, suggesting that the suppression of GM-CSF output could also be mediated through this receptor subtype (Sturzebecher *et al*., 1986; Stanford *et al*., 2000). Although IP-receptor antagonists are currently unavailable, the knowledge that HASM cells express IP-receptors (Clarke, D.L., Belvisi, M.G., Smith, S.J., Yacoub, M.H., Meja, K.K., Newton, R., Slater, D.M., Giembycz, M.A. – manuscript under review) and that cicaprost is a potent and selective IP-receptor agonist (Abramovitz *et al*., 2000; Dong *et al*., 1986) with weak activity at the human EP$_2$-receptor subtype (Breyer *et al*., 2001) supports this conclusion. Moreover, a role for EP$_4$-receptors can be excluded on the basis that although cicaprost binds with nanomolar affinity to this subtype (Abramovitz *et al*., 2000), neither AH 23848B nor L-161,982 antagonised the inhibitory effect of PGE2, ONO-AE1-259 and 16,16-dimethyl-PGE2 on GM-CSF release. At high concentrations ($>1\,\mu$M) of cicaprost, the second component of the response curve became evident (IC$_{50}$ $\sim 20\,\mu$M), which, based on the aforementioned discussion, may involve the EP$_2$-subtype.

In conclusion, PGE$_2$ inhibited GM-CSF release from IL-1$\beta$-stimulated HASM cells by acting through prostanoid receptors of the EP$_2$-subtype and this effect was mediated by PKA. Evidence is also provided that agonism of IP-receptors may also negatively regulate the expression of the *CSF2* gene. Finally, H-89 did not reproduce the effect of PKIα, possibly due to its ability to inhibit other protein kinases (Davies *et al*., 2000), and should not be considered a selective inhibitor of PKA.

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