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ORIGINAL ARTICLE **Basic Mechanisms in Allergic Disease**

Characterization of the EP receptor subtype that mediates the inhibitory effects of prostaglandin E_2 on IgE-dependent secretion from human lung mast cells

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Summary

Background Prostaglandin E_2 (PGE_2) has been shown to inhibit IgE-dependent histamine release from human lung mast cells. This effect of PGE_2 is believed to be mediated by EP_2 receptors. However, definitive evidence that this is the case has been lacking in the absence of EP_2-selective antagonists. Moreover, recent evidence has suggested that PGE_2 activates EP_4 receptors to inhibit respiratory cell function.

Objective The aim of this study was to determine the receptor by which PGE_2 inhibits human lung mast cell responses by using recently developed potent and selective EP_2 and EP_4 receptor antagonists alongside other established EP receptor ligands.

Methods The effects of non-selective (PGE_2, misoprostol), EP_2-selective (ONO-AE1-259, AH13205, butaprost-free acid) and EP_4-selective (L-902,688, TCS251) agonists on IgE-dependent histamine release and cyclic-AMP generation in mast cells were determined. The effects of EP_2-selective (PF-04418948, PF-04852946) and EP_4-selective (CJ-042794, L-161,982) antagonists on PGE_2 responses of mast cells were studied. The expression of EP receptor subtypes was determined by RT-PCR.

Results Prostaglandin E_2, EP_2 agonists and EP_4 agonists inhibited IgE-dependent histamine release from mast cells. PGE_2 and EP_2 agonists, but not EP_4 agonists, increased cyclic-AMP levels in mast cells. EP_4-selective antagonists did not affect the PGE_2 inhibition of histamine release, whereas EP_2-selective antagonists caused rightward shifts in the PGE_2 concentration–response curves. RT-PCR studies indicated that mast cells expressed EP_2 and EP_4 receptors.

Conclusions and Clinical Relevance Although human lung mast cells may express both EP_2 and EP_4 receptors, the principal mechanism by which PGE_2 inhibits mediator release in mast cells is by activating EP_2 receptors.

Keywords AH13205, butaprost, CJ-042794, EP receptors, L-161,982, L-902,688, mast cells, misoprostol, ONO-AE1-259, PF-04418948, PF-04852946, PGE_2, TCS251
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Introduction

Prostaglandin E_2 (PGE_2) is a highly pluripotent prostanoid displaying a wide range of effects in a variety of tissues. These include smooth muscle relaxation and contraction, inhibition of gastric acid secretion and both pro- and anti-inflammatory properties [1–3]. These effects of PGE_2 are principally mediated through EP receptors of which four have been identified [3–5]. EP_1 receptors cause elevations in intracellular calcium, EP_2 and EP_4 receptors mediate increases in cyclic-AMP through activation of adenylyl cyclase, whereas EP_3 receptors have been shown to inhibit and to activate adenylyl cyclase as well as to drive calcium mobilization [3–7]. Agonists and antagonists to these receptors have been developed, although achieving potency and selectivity has been challenging [8–11].

Targeting EP receptors has been mooted as a potential mechanism to treat asthma and other respiratory diseases. Clinical studies show that PGE_2 acts as a bronchodilator [12–14]. However, this beneficial effect of PGE_2 is complicated by the finding that PGE_2 also induces cough

[15]. The beneficial and undesirable effects of PGE_2 appear to be mediated by different EP receptors. It follows that dissociating bronchodilation from cough should be possible by using EP receptor–selective ligands. Cough appears to be mediated by EP_3 receptors [15], whereas the EP_2 receptor has been shown to mediate relaxation of human airways [16]. However, several very recent reports indicate that the EP_4 receptor, rather than the EP_2, might mediate bronchodilation by PGE_2 [17, 18].

In the context of treating respiratory diseases, an attendant potential benefit of PGE_2 might be to attenuate inflammatory responses in the lung [19]. In this regard, PGE_2 has been shown to inhibit human lung mast cell responses both *in vitro* [20–22] and *in vivo* [23]. Our own data suggest that this may be through activation of EP_2 receptors [21]. This conclusion was based on the fact that a purported EP_2-selective agonist, butaprost, also inhibited mast cell responses. Moreover, studies with an apparent EP_2-selective antagonist, AH6809, demonstrated that this compound antagonized the inhibitory effects of PGE_2 in mast cells. This conclusion is tempered by the knowledge that AH6809 is a weak and non-selective EP_2 receptor antagonist [9, 11]. The possibility exists, therefore, that EP receptors other than or in addition to EP_2 might contribute to the inhibitory effects of PGE_2 on human lung mast cells.

The aim of this study was to reappraise the effects of PGE_2 in human lung mast cells utilizing superior pharmacological probes that have emerged either very recently or within the last few years, including PF-04418948 the first potent and selective EP_2 receptor antagonist reported [24]. To this end, a wide range of EP agonists, EP_2-selective and EP_4-selective antagonists were studied to evaluate the expression of EP receptors by mast cells.

Methods

Buffers

Phosphate buffered saline (PBS) contained (mM): NaCl 137; $Na_2HPO_4{\cdot}12H_2O$ 8; KCl 2.7 and KH_2PO_4 1.5. PBS-HSA was PBS additionally supplemented with: $CaCl_2{\cdot}2H_2O$ 1 mM; $MgCl_2{\cdot}6H_2O$ 1 mM; glucose 5.6 mM and human serum albumin (HSA) 30 μg/mL. Standard imaging solution used for calcium imaging contained (mM): NaCl 147; HEPES 10; glucose 16; KCl 2; $CaCl_2{\cdot}2H_2O$ 2 and $MgCl{\cdot}6H_2O$ 1 to which bovine serum albumin (BSA, 1 mg/mL) was also added. The pH of all buffers was titrated to 7.3.

Preparation of compounds

Stock solutions (10 mM) of PGE_2, misoprostol, AH13205, 17-phenyl-trinor-PGE_2, butaprost-free acid, sulprostone, L-902,688 and TCS251 were prepared in ethanol and stored at −20°C. ONO-AE1-258 was prepared as a stock solution (10 mM) in distilled water and stored frozen in appropriate aliquots. All the following antagonists, PF-04418948, PF-04852946, CJ-042794 and L-161,982 were prepared as stock solutions (10 mM) in dimethyl sulphoxide and stored at −20°C. Stock solutions (10 mM) of (-)-isoprenaline bitartrate were prepared in 0.05% sodium metabisulphite (dissolved in 0.9% NaCl) and stored at 4°C. Fura-2-acetoxymethyl ester (fura-2-AM) was prepared as a stock solution (1 mM) in dimethyl sulphoxide. Monoclonal anti-human IgE (2 mg/mL stock) was stored frozen over the long term, but kept at 4°C for up to a month.

Lung tissue

Non-lesional tissue from lung resections of patients was obtained following surgery. Most of the patients were undergoing surgery for carcinoma. The male-to-female split was 2 to 1 and 90% of the patients were white Caucasians. The provision of lung tissue and the use of the tissue in this study were approved by the National Research Ethics' Committee (REC reference: 10/H1010/50). Written informed consent was obtained from those who permitted us to use tissue in this study.

Cell isolation

Mast cells were isolated from human lung tissue using methods described in detail elsewhere [25]. In brief, macroscopically normal tissue from lung resections was disrupted physically and enzymatically (collagenase Ia) to generate a mixed cell suspension of which 3–13% of the cells were mast cells. Mast cells were visualized by microscopy using an alcian blue stain [26]. This method generated approximately 6×10^5 mast cells per g of tissue. Mast cells prepared in this manner were used in histamine release experiments.

Mast cells of enhanced purity (10–35% purity) were generated by flotation over discontinuous Percoll gradients [27]. Mast cells were further purified using Dynabeads (Invitrogen, Paisley, UK) or a MACS magnetic cell sorting system (Miltenyi Biotec, Surrey, UK) according to the manufacturers' instructions. Mast cell yields using these methods were > 70% of the starting population and purities ranged from 88 to 100%. These purified cells were used in the cyclic-AMP assays and measurements of intracellular calcium. Mast cells of very high purity (≥ 95%) were used in RT-PCR experiments.

When cells were stored overnight they were incubated at a density of 0.1×10^6 mast cells per ml in 6-well plates in Rosswell Park Memorial Institute (RPMI)-1640 buffer supplemented with penicillin

(10 Units/mL), streptomycin (10 μg/mL), gentamicin (50 μg/mL), fungizone (1 μg/mL) and Foetal Bovine Serum (FBS; 10%).

Mediator release

Mediator release experiments were performed in PBS-HSA buffer. Anti-IgE was used to activate the mast cells. Human lung mast cells express IgE endogenously and so respond to anti-IgE without the need for a passive sensitization. Mast cells were incubated with or without an EP agonist for 10 min before challenge with a maximal releasing concentration of anti-IgE (2 μg/mL). In studies involving antagonists, mast cells were first incubated (50 min) with the antagonist and then together with agonist for a further 10 min before challenge with anti-IgE. Stimulus-induced secretion was allowed to proceed for 25 min at 37°C after which time the cells were pelleted by centrifugation (480 **g**, room temperature, 4 min). Histamine released into the supernatant was determined by the modified [28], automated fluorometric method of Siraganian [29]. Total histamine content was determined by lysing aliquots of the cells with perchloric acid at a final concentration of 1.6%. Cells incubated in buffer alone served as a measure of spontaneous histamine release which ranged from 2–8% of the total histamine content. Histamine release was thus expressed as a percentage of the total histamine content after subtracting the spontaneous histamine release. All experiments were performed in duplicate.

Assays for cyclic-AMP

Total cell cyclic-AMP levels were monitored according to methods that have been described elsewhere [30]. Purified cells were incubated (10 min) without or with PGE_2 or alternative agonists and the reaction terminated by the addition of ice-cold acidified ethanol and snap freezing of samples in liquid nitrogen. After thawing, samples were pelleted by centrifugation, supernatants saved and the ethanol evaporated using a rotary evaporator. Samples were reconstituted in assay buffer and cyclic-AMP levels were determined by using commercially available enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA).

Calcium imaging experiments

For calcium imaging experiments, mast cells were plated onto glass coverslips pre-coated with 0.1% poly-L-lysine hydrobromide and incubated for 30 min at 37°C to allow attachment before flooding with Dulbecco's Minimal Essential Medium (DMEM) supplemented with FBS (10%) and BSA (0.1%). Cells were then loaded with fura-2 AM (1 μM) for 30 min at 37°C and washed (15 min) with the supplemented DMEM, followed by a further wash (15 min) in standard imaging solution (see buffers).

Coverslips were placed in a superfusion chamber and mounted on an inverted microscope (Axiovert S100 TV, Zeiss, Cambridge, UK) equipped with a 40× oil immersion objective (NA 1.3, Zeiss). Cells were alternately illuminated at 340 and 380 nm with a 20 ms exposure time using a monochromator (Polychrome IV, TILL Photonics, Munich, Germany). Emitted light was passed through a 510-nm band pass filter and collected by a 512B Cascade CCD camera (Photometrics, Tucson, AZ, USA). Images were acquired at 0.5 Hz using MetaMorph® Meta imaging software (Molecular Devices, Sunnyvale, CA, USA). The recording chamber was continually superfused with standard imaging solution at a rate of approximately 1.5 mL/min. Anti-IgE and PGE_2, at the desired final concentration, were applied directly through the superfusion system. Experiments were performed at room temperature (21°C).

MetaMorph® Meta imaging software was used to analyse all calcium imaging experiments. A region of interest (ROI) was placed over each cell in the field of view on the 340 nm and 380 nm images and raw fluorescence values along with background fluorescence values were determined for each wavelength. Background fluorescence corresponding to the same frame was subtracted from the fluorescence value for each cell for both the 340 nm and 380 nm signals. The background-subtracted 340-nm fluorescence values were then divided by the background-subtracted 380-nm fluorescence values to give '$F_{340/380}$'. In all experiments, coverslips were prepared in triplicate and data for individual cells were taken from these triplicate coverslips and collated.

RT-PCR

RNA was extracted from purified mast cells (1–5 × 10^6 cells) by adding Tri-Reagent (1 mL) as instructed by the manufacturer (Sigma, Poole, UK). Contaminating DNA was then removed by DNase I (RNase free) digestion according to the manufacturer's instructions (Life Technologies, Paisley, UK). cDNA was then synthesized using the high-capacity cDNA reverse transcriptase kit (Life Technologies) from 1 μg total RNA as directed by the manufacturer.

The cDNA was then amplified using primers specific to regions of the human EP receptor subtypes by PCR using conditions and methods that have been described elsewhere [31, 32]. Expression of the housekeeping gene, β-actin, was also evaluated. Primers were synthesized by Sigma (Poole, UK). The primer pairs that were used are provided in Table 1.

Table 1. Primers used to determine EP receptor expression by RT-PCR

Target	Primers	Annealing conditions	Fragment size (bp)
EP_1	5′-ATCATGGTGGTGTCGTGCAT-3′ (sense) 5′-TACACCCAAGGGTCCAGGAT-3′ (antisense)	56°C, 30 s	149
EP_2	5′-CAACCTCATCCGCATGCAC-3′ (sense) 5′-CTCAAAGGTCAGCCTG-3′ (antisense)	55°C, 30 s	419
EP_3	5′-CGCCTCAACCACTCCTACACA-3′ (sense) 5′-GCAGACCGACAGCACGCACAT-3′ (antisense)	61°C, 30 s	837
EP_4	5′-TGGTATGTGGGCTGGCTG-3′ (sense) 5′-GAGGACGGTGGCGAGAAT-3′ (antisense)	55°C, 30 s	434
β-actin	5′-ATATCGCCGCGCTCGTCGTC-3′ (sense) 5′-TAGCCGCGCTCGGTGAGGAT-3′ (antisense)	60°C, 45 s	583

The PCR was carried out using GoTAQ® Flexi DNA polymerase (Promega, Southampton, UK) with 1 μL cDNA, both primers (200 nM of each primer) and $MgCl_2$ (1 mM) in a final volume of 25 μL. Cycling conditions were 95°C for 1 min, 95°C for 15 s, see Table 1 for annealing conditions, 72°C for 90 s, with a final elongation step of 72°C for 10 min. All PCR reactions consisted of 35 or 40 cycles. PCR products (5 μL) were visualized with ethidium bromide on 2% agarose gels alongside Quick-Load® 100 bp DNA ladder (New England Biolabs, Hitchin, UK).

To ensure that the correct amplification had taken place, PCR products were cut from the agarose gel, cleaned using a MinElute Gel Extraction Kit (Qiagen, Crawley, UK), according to the manufacturer's instructions and then subjected to in-house genotypic analysis by automated sequencing (ABI 3730 DNA Analyser; Applied Biosystems, Carlsbad, CA, USA).

Materials

The following were purchased from the sources indicated; AH13205, BSA, sulprostone, PGE_2, collagenase, HSA, isoprenaline, Percoll, poly-L-lysine hydrobromide, Tri-Reagent (all Sigma, Poole, UK); 17-phenyl-trinor-PGE_2 (Biomol, Plymouth Meeting, PA, USA); fura-2-AM, gentamicin, penicillin/streptomycin, fungizone, RPMI 1640, DMEM (Invitrogen, Paisley, UK); butaprost (free acid), misoprostol (free acid), L-902,688 (Cayman Chemical Company, Ann Arbor, MI, USA); L-161,982, TCS251 (Tocris Bioscience, Bristol, UK); anti-human IgE, clone HP6061 (Stratech Scientific Ltd, Newmarket, UK); JW8-IgE (BioServ UK Ltd, Sheffield, UK).

PF-04418948, PF-04852946 and CJ-042794 were obtained from Pfizer Global Research & Development (Sandwich, UK). PF-04418948 will shortly be available commercially from Sigma-Aldrich, Tocris and Toronto Research Chemicals Inc. (North York, ON, Canada). ONO-AE1-259 was a kind gift from Ono Pharmaceutical Company Ltd (Osaka, Japan). LAD2 cells were a kind gift from Dr Dean Metcalfe, Laboratory of Allergic Diseases, National Institutes of Health (Bethesda, MD, USA).

Data analysis

Antagonist affinity (pK_B) was determined either by performing Schild analysis or by using the Gaddum equation [33]. In Schild analysis, a graph of log(dose ratio − 1) was plotted against log(antagonist concentration), the intercept on the *x*-axis providing a value for pK_B. The dose ratio was calculated as the ratio of the concentration of agonist required to produce an equivalent inhibitory effect in the presence and absence of antagonist. Alternatively, antagonist affinity was estimated using the Gaddum equation: pK_B = log(dose ratio − 1) − log(antagonist concentration). Maximal responses (E_{max}) and potencies (EC_{50}) were determined by non-linear regression analysis (GraphPad Prism, version 5.0d, La Jolla, CA, USA). Statistical significance was performed on the raw data utilizing repeated measures ANOVA.

Results

Functional studies with agonists

In agreement with previous studies, PGE_2 was found to inhibit IgE-dependent histamine release from human lung mast cells in a concentration-dependent manner (Fig. 1a). In these same experiments, the effects of the non-selective EP agonist, misoprostol, were also studied. Misoprostol (EC_{50}; 4.0 μM) was an effective inhibitor of histamine release, but was about threefold less potent than PGE_2 (EC_{50}; 1.6 μM).

The effects of several EP_2-selective agonists were studied. Butaprost (free acid form) inhibited IgE-dependent histamine release concentration dependently, but was about eightfold less potent than PGE_2, and among experiments, maximal responses were not

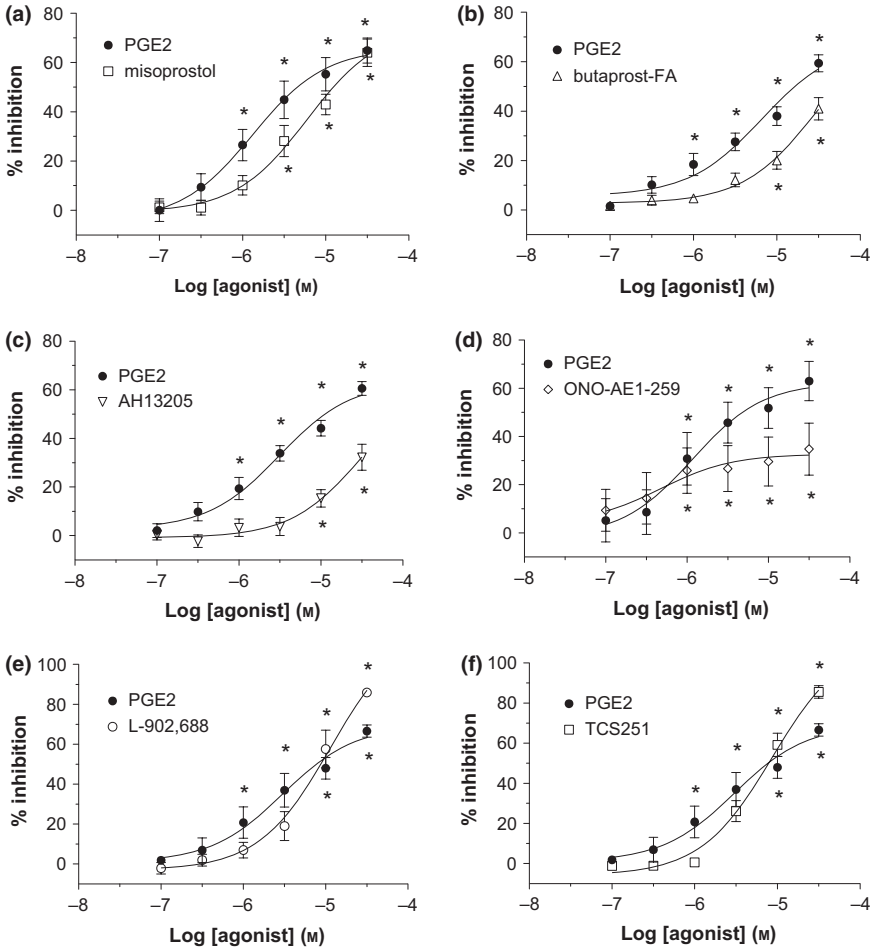

Fig. 1. Effects of EP agonists on mast cells. Cells were incubated with or without PGE_2 and (a) misoprostol, (b) butaprost (free acid), (c) AH13205, (d) ONO-AE1-259, (e) L-902,688 or (f) TCS251 for 10 min before challenge with anti-IgE (2 μg/mL) for 25 min for histamine release. Values are expressed as the % inhibition of the control unblocked histamine releases which ranged from 28 ± 5% to 34 ± 5%. Values are means ± SEM, for 5–11 experiments. Statistically significant ($P < 0.05$ at least) levels of inhibition compared with unblocked control levels are indicated by an asterisk.

observed consistently over the concentration range used (Fig. 1b). Two further EP_2-selective agonists were studied, AH13205 (Fig. 1c) and ONO-AE1-259 (Fig. 1d). AH13205 inhibited IgE-dependent histamine release modestly. ONO-AE1-259 was a relatively potent inhibitor (EC_{50}; 0.32 μM) of histamine release, but appeared to behave as a partial agonist.

Two EP_4 agonists were also studied, L-902,688 and TCS251. Both compounds inhibited IgE-dependent histamine release. Both compounds were less potent (EC_{50}; ~ 6 μM) than PGE_2, but displayed greater maximal activity than PGE_2 (Figs 1e,f). In keeping with our previous studies [21], neither sulprostone, an EP_1/EP_3-selective agonist, nor 17-phenyl-trinor-PGE_2, an EP_1-selective agonist, had any effect on human lung mast cells (data not shown).

Taken together, the results from the agonist studies confirm that PGE_2 inhibits histamine secretion from human lung mast cells and, based on the pharmacology of the agonists used in this study, this effect may be mediated by both EP_2 and EP_4 receptors.

Cyclic-AMP studies

The effects of PGE_2 and EP agonists on cyclic-AMP levels in mast cells were determined. PGE_2 was effective at increasing total cell cyclic-AMP in mast cells (Fig. 2). The non-selective EP agonist, misoprostol, was almost as effective as PGE_2 at elevating cyclic-AMP levels. The EP_2-selective agonists, ONO-AE1-259, butaprost and AH13205 all increased mast cell cyclic-AMP to a statistically significant ($P < 0.05$) extent, but were about a third as effective as PGE_2. By contrast, the EP_4-selective agonists L-902,688 and TCS251 were completely ineffective at increasing cyclic-AMP. Similarly, 17-phenyl-trinor-PGE_2 (EP_1-selective) and sulprostone

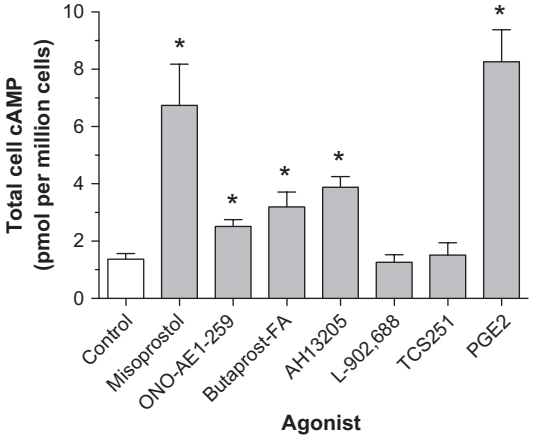

Fig. 2. Effects of Prostaglandin E_2 (PGE_2) and other EP agonists on cyclic-AMP levels in mast cells. Mast cells were incubated without or with PGE_2 (30 μM) or other EP agonists (30 μM) for 10 min. After this incubation, the cells were snap frozen, solubilized and total cell cyclic-AMP levels measured. Mast cell purities ranged from 90 to 95%. Values are means ± SEM for 4–6 experiments. Statistically significant ($P < 0.05$ at least) increases in cyclic-AMP over unstimulated control levels are indicated by an asterisk.

(EP_1/EP_3-selective) were ineffective at enhancing mast cell cyclic-AMP levels (data not shown).

Calcium studies

The effects of PGE_2 on intracellular calcium changes were investigated. In these experiments the effects of anti-IgE challenge of mast cells on intracellular calcium change were also evaluated. Anti-IgE induced a time-dependent increase in intracellular calcium that was maximal 3 min after application of anti-IgE (Fig. 3a). By contrast, PGE_2 on its own had no effect on intracellular calcium in mast cells and, moreover, PGE_2 did not affect the increase in calcium signals driven by anti-IgE (Fig. 3b).

Studies with antagonists

That PGE_2 enhanced cyclic-AMP levels but had no effect on intracellular calcium suggests that PGE_2 acts on mast cells through either EP_2 or EP_4 receptors. To evaluate this further, potent and selective antagonists of EP_2 and EP_4 receptors were studied.

The effects of the antagonists PF-04418948 (EP_2-selective) and CJ-042794 (EP_4-selective) were investigated [24, 34]. Mast cells were incubated with either PF-04418948 (300 nM) or CJ-042794 (1 μM) or both antagonists before incubation with PGE_2 and then challenged with anti-IgE (Fig. 4a). PF-04418948 effectively antagonized the PGE_2 elicited inhibition of histamine release. No antagonism of the PGE_2 inhibition was seen by CJ-042794 when used either alone or in

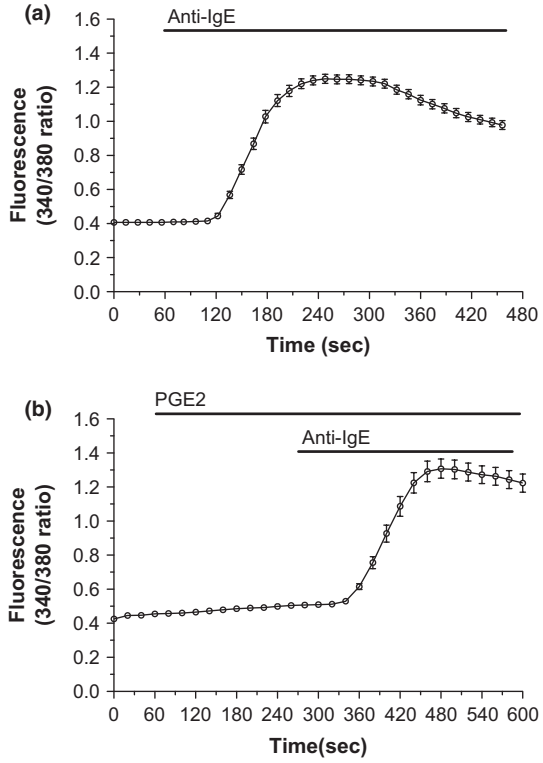

Fig. 3. Effects of PGE_2 on intracellular calcium in mast cells. Mast cells were loaded with fura-2-AM and then time-dependent changes in intracellular calcium were determined following incubation of cells with (a) anti-IgE (2 μg/mL) or (b) PGE_2 (10 μM) before challenge with anti-IgE (2 μg/mL). Fluorescence values are based on data from (a) 68 and (b) 42 mast cells from 1 donor. Values are means ± SEM. This experiment was performed on mast cells isolated from 3 different donors with identical outcomes. Mast cell purities were ≥ 99%.

combination with PF-04418948. Similar results were observed with PF-04418948 and CJ-042794 when misoprostol was used as the agonist (Fig. 4b). The EP_4-selective antagonist, L-161,982 (2 μM), was also studied and, in keeping with data obtained with CJ-042794, L-161,982 was found to be ineffective as an antagonist (data not shown).

The nature of the antagonism was assessed by Schild regression analysis [33]. For this, the effects of increasing concentrations of PF-04418948 (30, 100 and 300 nM) on the PGE_2 inhibition were determined. The data demonstrated that increasing concentrations of the antagonist led to progressive rightward shifts in the concentration response curve to PGE_2 (Fig. 5a). Schild analysis (Fig. 5b) indicated a pK_B value for PF-04418948 of 7.55 (K_B, 28 nM) and a slope of 0.92 which is close to unity suggesting that PF-04418948 is acting as a competitive antagonist [33]. An estimate of the pK_B of PF-04418948 based on antagonism of the inhibition of mediator release by misoprostol (data not shown) was found to be 7.44 (K_B, 36 nM).

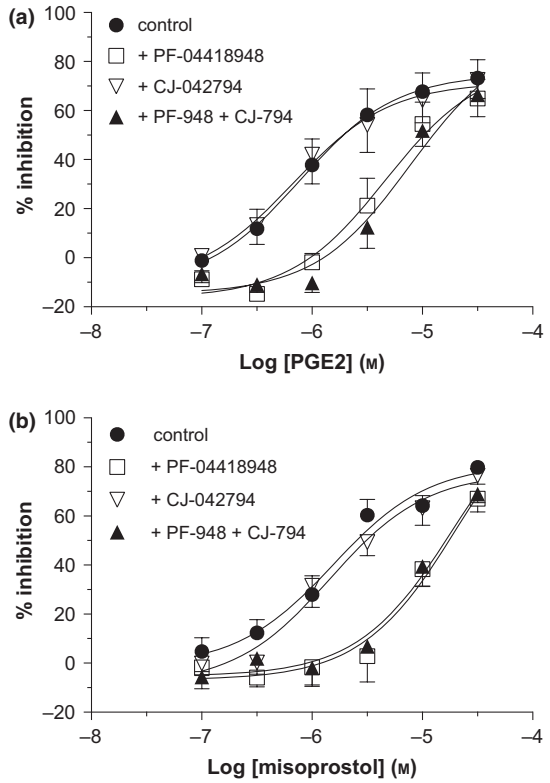

Fig. 4. Effect of EP receptor antagonists on (a) PGE_2 and (b) misoprostol. Mast cells were incubated (50 min) without or with the EP_2-selective antagonist PF-04418948 (300 nM) or the EP_4-selective antagonist CJ-042794 (1 μM) or both antagonists and then without or with PGE_2 or misoprostol for 10 min before challenge with anti-IgE (2 μg/mL) for 25 min. Values are expressed as the % inhibition of control histamine release which ranged from 25 ± 8 to 28 ± 9% for (a) and from 25 ± 3 to 26 ± 4% for (b). Values are means ± SEM for 4 and 5 experiments for (a) and (b) respectively.

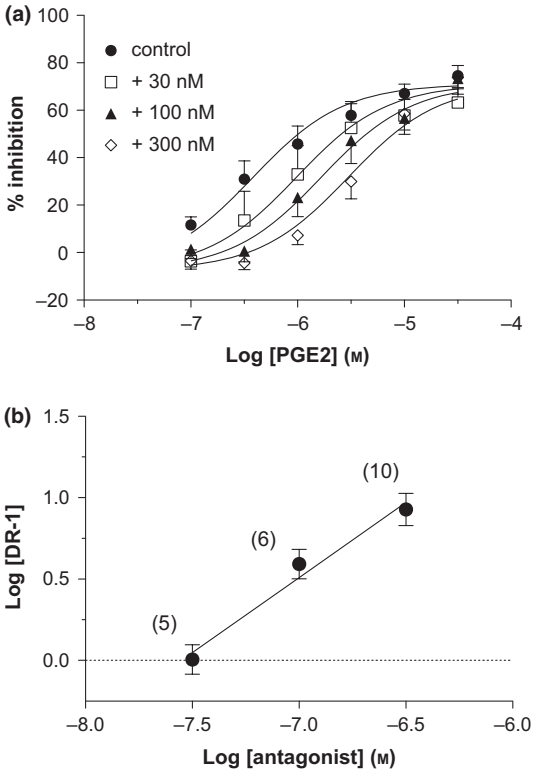

Fig. 5. Schild regression analysis. (a) Mast cells were incubated (50 min) without or with increasing concentrations of the EP_2-selective antagonist PF-04418948 and then without or with PGE_2 for 10 min before challenge with anti-IgE (2 μg/mL) for 25 min. Values are expressed as the % inhibition of control histamine release which ranged from 25 ± 6 to 28 ± 7%. Values are means ± SEM for 5 experiments. (b) Dose ratios (DR) were calculated from the data in (a) as well as from other experiments where PF-04418948 had been used to antagonize the effects of PGE_2. A Schild plot of log(DR-1) vs. log (antagonist) was constructed. The slope of the regression was 0.92 and the calculated pK_B, 7.55. Values in brackets indicate n values for each point.

An alternative EP_2-selective antagonist, PF-04852946, structurally distinct from PF-04418948, was also studied. PF-04852946 is about tenfold more potent than PF-04418948 at EP_2 receptors (see Table S1). PF-04852946 was studied and found to be an effective antagonist (see Fig. S1). pK_B estimates for PF-04852946 were 8.53 (K_B, 3.0 nM) and 8.45 (K_B, 3.5 nM) for the antagonism of PGE_2 and misoprostol respectively.

RT-PCR studies

RT-PCR was used to determine the pattern of expression of EP receptors by human lung mast cells and LAD2 cells, a human mast cell line [35]. The data indicated that human lung mast cells express message for EP_2 and EP_4 receptors, whereas LAD2 cells do not express either of these receptors, but do express message for EP_3 receptors (Fig. 6).

Additional studies were performed to determine whether PGE_2 had any inhibitory effects on histamine release from LAD2 cells. LAD2 cells were passively sensitized (20 h) with IgE (clone JW8, ~ 0.2 μg/mL), the cells washed and then incubated (10 min) with PGE_2 (10^{-7} – 3×10^{-5} M) before challenge (25 min) with anti-IgE (2 μg/mL). PGE_2 had no inhibitory effects on the IgE-dependent release of histamine from LAD2 cells, whereas in the same experiments the β-adrenoceptor agonist, isoprenaline (10^{-10} – 10^{-5} M), was an effective inhibitor (data not shown).

Discussion

The purpose of these studies was to determine which EP receptor mediates the effects of PGE_2 on human lung mast cells. Previous studies of ours suggested that PGE_2 works through EP_2 receptors to stabilize mast cells [21]. However, this conclusion was drawn largely on the basis of studies utilizing antagonists of EP_2 and EP_4

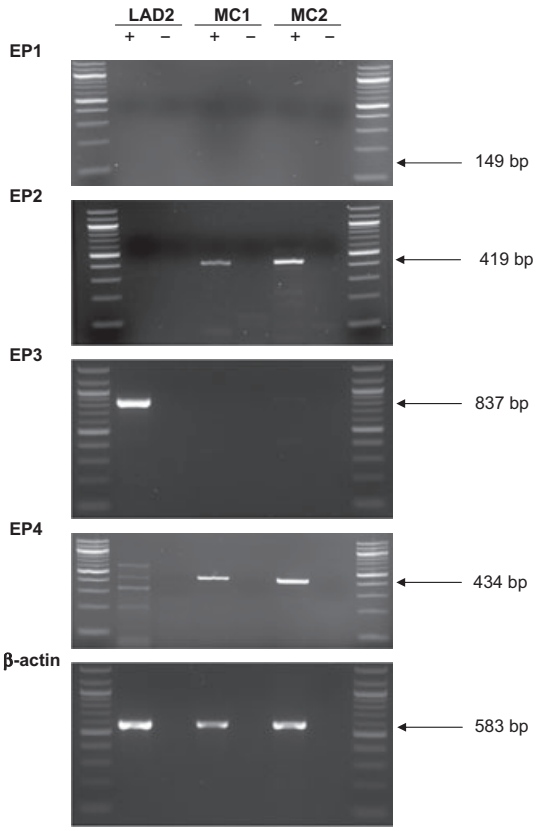

Fig. 6. EP receptor expression in mast cells and LAD2 cells. Isolated RNA was converted into cDNA by reverse transcriptase (+) and, as a control, this reaction step was also carried out in the absence of reverse transcriptase (−). Amplification of cDNA was performed using primers specific for each of the EP receptor subtypes and β-actin. Expression profiles for two mast cell preparations (MC1 and MC2) and LAD2 cells are shown. No EP_1 was detected in either cell type, but in separate experiments the presence of EP_1 could be readily demonstrated in several breast cancer cell lines, MDA-MB-468, MDA-MB-231 and ZR-75-1 [32]. These findings are representative of three different mast cell preparations. Lanes at either end of each gel represent a 100 bp ladder. The purity of mast cells was ≥ 97%.

receptors that lacked potency and selectivity. Moreover, recent studies in human bronchial smooth muscle suggest that the EP_4 receptor [17, 18], and not as previously thought the EP_2 receptor [16], mediates PGE_2-dependent bronchodilation. This has led to the suggestion that the EP_4 receptor could serve as a novel target for respiratory diseases [17, 18]. For these reasons, we have reappraised the actions of PGE_2 on human lung mast cells utilizing superior pharmacological probes, some of which have only become available very recently.

In agreement with previous studies [20–22], PGE_2 was an effective inhibitor of IgE-dependent histamine release from human lung mast cells. The effects of a range of additional EP agonists on histamine release were also studied. Misoprostol, an analogue of PGE_1 that is used clinically to prevent gastric acid secretion and to induce labour [36, 37], was also an effective inhibitor of histamine release. Misoprostol was about threefold less potent than PGE_2 in mast cells. Misoprostol is a relatively non-selective agonist that is known to exert effects at EP_2, EP_3 and EP_4 receptors but not at EP_1 receptors. Moreover, it has been reported that misoprostol is about 30-fold less potent than PGE_2 at EP_3 and EP_4 receptors, but about sevenfold less potent than PGE_2 at EP_2 receptors [9]. The relative potencies of misoprostol and PGE_2 imply that mast cells are likely to express EP_2 receptors.

A response to butaprost is considered diagnostic for the functional expression of EP_2 receptors [8]. In this study, butaprost (free acid) inhibited histamine release, but was about eightfold less potent than PGE_2. Further studies were performed with ONO-AE1-259 which has been reported to be a potent and selective agonist at EP_2 receptors [38–40]. Our studies indicated that ONO-AE1-259 showed quite variable activity and although it was more potent it was a partial agonist relative to PGE_2. AH13205, which is also recognized as an EP_2 agonist [41], was also studied in this system and was found to be the least potent of all the EP agonists tested. Several EP_4 agonists were also studied, L-902,688 and TCS251 [18], and although both compounds inhibited histamine release from mast cells, possibly suggesting that mast cells express EP_4 receptors, the concentration response curves were steep and maximal inhibition was greater than that seen with PGE_2.

In additional studies, the effects of PGE_2 on total cyclic-AMP levels in mast cells were evaluated. As the EP_2 receptor is coupled to adenylyl cyclase [10, 42], the demonstration that PGE_2, and misoprostol, elevated cyclic-AMP provides further evidence that the EP_2 receptor is expressed by mast cells. However, as the EP_4 receptor is also coupled to adenylyl cyclase [10] these data do not exclude the possible expression of EP_4 receptors by mast cells. It was of interest that EP_2-selective agonists, butaprost, ONO-AE1-259 and AH13205 raised cyclic-AMP in mast cells, whereas the EP_4-selective agonists, L-902,688 and TCS251, were ineffective. These data provide further evidence that PGE_2 acts through EP_2 receptors to attenuate mast cell responses.

Previous reports have indicated that cultured human mast cells, derived from either cord blood or peripheral blood, express EP_3 receptors that, when activated, cause elevations in intracellular calcium [43] or enhance antigen-driven calcium signals, potentiating mast cell secretion albeit in a donor-dependent fashion [44]. These findings contrast with this present study in human lung mast cells as no PGE_2-mediated changes in calcium signalling were observed, arguing against the functional expression of either EP_3 or EP_1 receptors in these cells. These findings are also supported by our

previous studies showing that neither sulprostone (EP_3 agonist) nor 17-phenyl-trinor-PGE_2 (EP_1 agonist) has any effect on IgE-dependent histamine release from mast cells [21]. The reason for this difference between cultured blood-derived mast cells and human lung mast cells is not obvious, although it is possible that expression of EP receptors in mast cells is pre-programmed in a tissue-dependent manner. Overall, these data further reinforce the notion that PGE_2 does not mediate effects through either EP_3 or EP_1 receptors in human lung mast cells.

Further characterization of EP receptors expressed by human lung mast cells was determined using EP-selective antagonists. Up until very recently the only EP_2 antagonist available for experimental use was AH6809 which is weak and non-selective [9, 11]. However, a recent report has described the development of a novel, potent and selective EP_2 receptor antagonist, PF-04418948 [24], and this antagonist was used in this study. PF-04418948 was found to be an effective antagonist of the PGE_2 inhibition of histamine release from mast cells. The antagonism was competitive and although the affinity ($K_B \sim 30$ nM) of PF-04418948 was about four- to fivefold lower than that reported for human myometrium [24], it is of interest that PF-04418948 showed very similar affinity when antagonizing the effects of misoprostol in mast cells. These data suggest that PGE_2 and misoprostol are acting at the same receptor in human lung mast cells.

In further support of this reasoning, an alternative EP_2 antagonist was also studied, PF-04852946, which in other systems has been shown to be tenfold more potent at the EP_2 receptor than PF-04418948 (see Supporting Information). It is noteworthy that when used in mast cells, the affinity of PF-04852946 ($K_B \sim 3$ nM) was about tenfold higher than that for PF-04418948. These data strongly indicate that both antagonists are acting at the same receptor to antagonize the effects of PGE_2 and that this is likely to be the EP_2 receptor.

By contrast, the EP_4-selective antagonists, CJ-042794 and L-161,982, failed to reverse the PGE_2 inhibition in human mast cells. Attempts to unveil a potential EP_4-mediated effect of PGE_2 by incubating mast cells with an EP_2- and an EP_4-selective antagonist at the same time, an approach that has been used in alternative systems [39], were also unsuccessful. These data reinforce the notion that the EP_2 receptor is the principal receptor through which PGE_2 mediates its inhibitory effects on histamine secretion in human lung mast cells.

An aspect of this study that is difficult to reconcile is the finding that EP_4 agonists were effective inhibitors of IgE-dependent histamine release. This inhibitory effect of EP_4 agonists was not accompanied by increases in total cell cyclic-AMP, suggesting that if EP_4 receptors are expressed by mast cells these may not be coupled to adenylyl cyclase. However, it should be noted that in experiments not shown in this study, the inhibitory effects of the EP_4 agonist, L-902,688, were unaffected by either EP_4- or EP_2-selective antagonists. These data suggest that the effects of L-902,688 on mast cells are not due to interactions with either EP_4 or EP_2 receptors and so a mechanism for this inhibition remains unexplained currently.

Confirmatory studies were performed to determine which EP receptors were expressed in mast cells. These experiments demonstrated the presence of mRNA transcripts for both EP_2 and EP_4 receptors, but no evidence for EP_1 or EP_3 receptors. At a functional level, an overwhelming body of evidence indicates that PGE_2 acts through EP_2 receptors to modulate mast cell responses. Despite the apparent expression of EP_4 receptors there was little evidence for EP_4 involvement functionally. It is possible, however, that EP_4 receptors can contribute to the effects of PGE_2 on mast cells, but not in the context of modulating mediator release. It is of interest that LAD2 cells, in marked contrast to human lung mast cells, appeared to express only EP_3 receptors, and PGE_2 had no inhibitory effects on IgE-dependent histamine release in these cells. These findings emphasize the heterogeneity that exists among mast cells derived from different sources [45].

In summary, this study provides definitive evidence that PGE_2 acts through EP_2 receptors to stabilize human lung mast cells. This has been determined, principally, using potent and selective EP_2 receptor antagonists that have only recently been developed. These probes are likely to be of particular use in a general sense for the characterization of EP_2 receptors in a variety of systems.

Clinical relevance

Targeting EP receptors has been suggested as a potential mechanism to treat asthma and other respiratory diseases. Recent studies have shown that the EP_4 receptor mediates bronchodilation and that targeting this receptor may be a novel therapeutic approach for bronchodilator therapy [17, 18]. However, our data indicate that an EP_4-targeted therapy is unlikely to attenuate mediator release from human lung mast cells.

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Conflicts of interest

Nick Pullen and Sarah Skerratt are employees of Pfizer Global Research & Development. Peter Peachell has received funding for research from Pfizer and this study was supported in part by Pfizer.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Structures and pharmacological profiles of key EP_2 and EP_4 receptor antagonists utilised in these studies.

Figure S1. Effect of the EP_2 receptor antagonist, PF-04852946, on (a) PGE_2 and (b) misoprostol. Mast cells were incubated (50 min) without or with PF-04852946 (30 nM) and then without or with PGE_2 or misoprostol for 10 min before challenge with anti-IgE (2 μg/mL) for 25 min. Values are expressed as the % inhibition of control histamine release which were 23 ± 4 and 25 ± 5% for (a) and 22 ± 3 and 23 ± 3% for (b). Values are means ± SEM for 5 and 4 experiments for (a) and (b), respectively.