

Characterization of the EP receptor subtype that mediates the inhibitory effects of prostaglandin E₂ on IgE-dependent secretion from human lung mast cells

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Summary

Background Prostaglandin E₂ (PGE₂) has been shown to inhibit IgE-dependent histamine release from human lung mast cells. This effect of PGE₂ is believed to be mediated by EP₂ receptors. However, definitive evidence that this is the case has been lacking in the absence of EP₂-selective antagonists. Moreover, recent evidence has suggested that PGE₂ activates EP₄ receptors to inhibit respiratory cell function.

Objective The aim of this study was to determine the receptor by which PGE₂ inhibits human lung mast cell responses by using recently developed potent and selective EP₂ and EP₄ receptor antagonists alongside other established EP receptor ligands.

Methods The effects of non-selective (PGE₂, misoprostol), EP₂-selective (ONO-AE1-259, AH13205, butaprost-free acid) and EP₄-selective (L-902,688, TCS251) agonists on IgE-dependent histamine release and cyclic-AMP generation in mast cells were determined. The effects of EP₂-selective (PF-04418948, PF-04852946) and EP₄-selective (CJ-042794, L-161,982) antagonists on PGE₂ responses of mast cells were studied. The expression of EP receptor subtypes was determined by RT-PCR.

Results Prostaglandin E₂, EP₂ agonists and EP₄ agonists inhibited IgE-dependent histamine release from mast cells. PGE₂ and EP₂ agonists, but not EP₄ agonists, increased cyclic-AMP levels in mast cells. EP₄-selective antagonists did not affect the PGE₂ inhibition of histamine release, whereas EP₂-selective antagonists caused rightward shifts in the PGE₂ concentration–response curves. RT-PCR studies indicated that mast cells expressed EP₂ and EP₄ receptors.

Conclusions and Clinical Relevance Although human lung mast cells may express both EP₂ and EP₄ receptors, the principal mechanism by which PGE₂ inhibits mediator release in mast cells is by activating EP₂ receptors.

Keywords AH13205, butaprost, CJ-042794, EP receptors, L-161,982, L-902,688, mast cells, misoprostol, ONO-AE1-259, PF-04418948, PF-04852946, PGE₂, TCS251

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Introduction

Prostaglandin E₂ (PGE₂) 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 PGE₂ are principally mediated through EP receptors of which four have been identified [3–5]. EP₁ receptors cause elevations in intracellular calcium, EP₂ and EP₄ receptors mediate increases in cyclic-AMP

through activation of adenylyl cyclase, whereas EP₃ 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 PGE₂ acts as a bronchodilator [12–14]. However, this beneficial effect of PGE₂ is complicated by the finding that PGE₂ also induces cough

[15]. The beneficial and undesirable effects of PGE₂ 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 EP₃ receptors [15], whereas the EP₂ receptor has been shown to mediate relaxation of human airways [16]. However, several very recent reports indicate that the EP₄ receptor, rather than the EP₂, might mediate bronchodilation by PGE₂ [17, 18].

In the context of treating respiratory diseases, an attendant potential benefit of PGE₂ might be to attenuate inflammatory responses in the lung [19]. In this regard, PGE₂ 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 EP₂ receptors [21]. This conclusion was based on the fact that a purported EP₂-selective agonist, butaprost, also inhibited mast cell responses. Moreover, studies with an apparent EP₂-selective antagonist, AH6809, demonstrated that this compound antagonized the inhibitory effects of PGE₂ in mast cells. This conclusion is tempered by the knowledge that AH6809 is a weak and non-selective EP₂ receptor antagonist [9, 11]. The possibility exists, therefore, that EP receptors other than or in addition to EP₂ might contribute to the inhibitory effects of PGE₂ on human lung mast cells.

The aim of this study was to reappraise the effects of PGE₂ 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 EP₂ receptor antagonist reported [24]. To this end, a wide range of EP agonists, EP₂-selective and EP₄-selective antagonists were studied to evaluate the expression of EP receptors by mast cells.

Methods

Buffers

Phosphate buffered saline (PBS) contained (mM): NaCl 137; Na₂HPO₄·12H₂O 8; KCl 2.7 and KH₂PO₄ 1.5. PBS-HSA was PBS additionally supplemented with: CaCl₂·2H₂O 1 mM; MgCl₂·6H₂O 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; CaCl₂·2H₂O 2 and MgCl₂·6H₂O 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 PGE₂, misoprostol, AH13205, 17-phenyl-trinor-PGE₂, 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 ($\geq 95\%$) 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 PGE₂ 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 PGE₂, 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 × 10⁶ 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'-ATCATGGTGGTGTGTCGTCAT-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'-CGCCTCAACCACTCTACACA-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 MgCl₂ (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, PGE₂, collagenase, HSA, isoprenaline, Percoll, poly-L-lysine hydrobromide, Tri-Reagent (all Sigma, Poole, UK); 17-phenyl-trinor-PGE₂ (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 (EC₅₀) 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, PGE₂ 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 (EC₅₀; 4.0 µM) was an effective inhibitor of histamine release, but was about threefold less potent than PGE₂ (EC₅₀; 1.6 µM).

The effects of several EP₂-selective agonists were studied. Butaprost (free acid form) inhibited IgE-dependent histamine release concentration dependently, but was about eightfold less potent than PGE₂, and among experiments, maximal responses were not

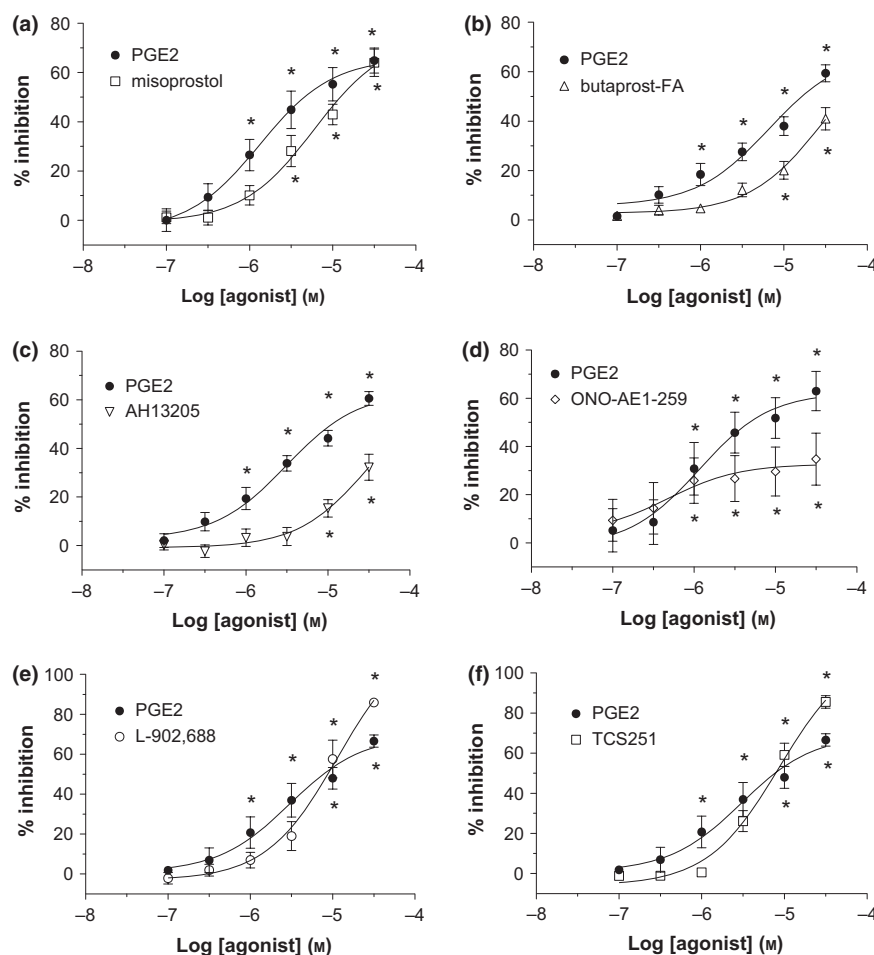


Fig. 1. Effects of EP agonists on mast cells. Cells were incubated with or without PGE₂ and (a) misoprostol, (b) butaprost (free acid), (c) AH13205, (d) ONO-AE1-259, (e) L-902,688 or (f) TCS251 for 10 min before challenge with anti-IgE (2 µg/mL) for 25 min for histamine release. Values are expressed as the % inhibition of the control unblocked histamine releases which ranged from $28 \pm 5\%$ to $34 \pm 5\%$. Values are means $\pm$ 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 EP₂-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 EP₄ 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 PGE₂, but displayed greater maximal activity than PGE₂ (Figs 1e,f). In keeping with our previous studies [21], neither sulprostone, an EP₁/EP₃-selective agonist, nor 17-phenyl-trinor-PGE₂, an EP₁-selective agonist, had any effect on human lung mast cells (data not shown).

Taken together, the results from the agonist studies confirm that PGE₂ 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 EP₂ and EP₄ receptors.

Cyclic-AMP studies

The effects of PGE₂ and EP agonists on cyclic-AMP levels in mast cells were determined. PGE₂ was effective at increasing total cell cyclic-AMP in mast cells (Fig. 2). The non-selective EP agonist, misoprostol, was almost as effective as PGE₂ at elevating cyclic-AMP levels. The EP₂-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 PGE₂. By contrast, the EP₄-selective agonists L-902,688 and TCS251 were completely ineffective at increasing cyclic-AMP. Similarly, 17-phenyl-trinor-PGE₂ (EP₁-selective) and sulprostone

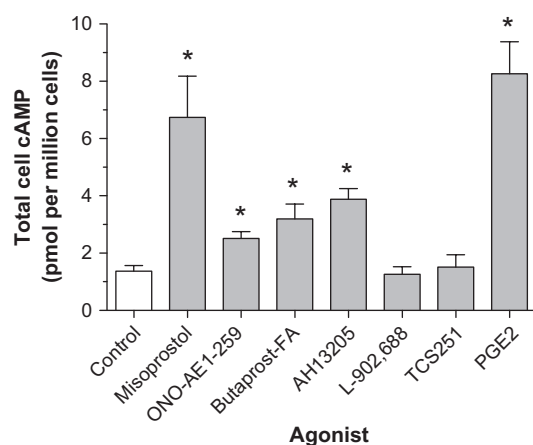


Fig. 2. Effects of Prostaglandin E₂ (PGE₂) and other EP agonists on cyclic-AMP levels in mast cells. Mast cells were incubated without or with PGE₂ (30 μ M) or other EP agonists (30 μ M) for 10 min. After this incubation, the cells were snap frozen, solubilized and total cell cyclic-AMP levels measured. Mast cell purities ranged from 90 to 95%. Values are means $\pm$ 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.

(EP₁/EP₃-selective) were ineffective at enhancing mast cell cyclic-AMP levels (data not shown).

Calcium studies

The effects of PGE₂ 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, PGE₂ on its own had no effect on intracellular calcium in mast cells and, moreover, PGE₂ did not affect the increase in calcium signals driven by anti-IgE (Fig. 3b).

Studies with antagonists

That PGE₂ enhanced cyclic-AMP levels but had no effect on intracellular calcium suggests that PGE₂ acts on mast cells through either EP₂ or EP₄ receptors. To evaluate this further, potent and selective antagonists of EP₂ and EP₄ receptors were studied.

The effects of the antagonists PF-04418948 (EP₂-selective) and CJ-042794 (EP₄-selective) were investigated [24, 34]. Mast cells were incubated with either PF-04418948 (300 nM) or CJ-042794 (1 μ M) or both antagonists before incubation with PGE₂ and then challenged with anti-IgE (Fig. 4a). PF-04418948 effectively antagonized the PGE₂ elicited inhibition of histamine release. No antagonism of the PGE₂ inhibition was seen by CJ-042794 when used either alone or in

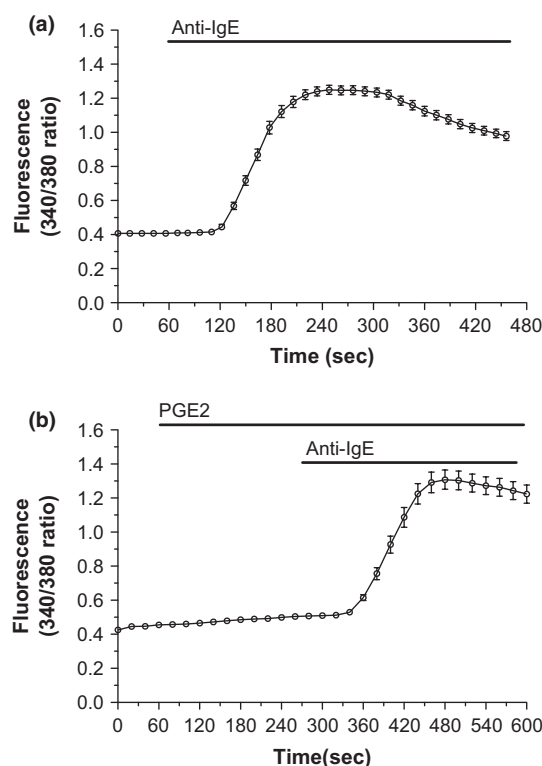


Fig. 3. Effects of PGE₂ on intracellular calcium in mast cells. Mast cells were loaded with fura-2-AM and then time-dependent changes in intracellular calcium were determined following incubation of cells with (a) anti-IgE (2 μ g/mL) or (b) PGE₂ (10 μ M) before challenge with anti-IgE (2 μ g/mL). Fluorescence values are based on data from (a) 68 and (b) 42 mast cells from 1 donor. Values are means $\pm$ SEM. This experiment was performed on mast cells isolated from 3 different donors with identical outcomes. Mast cell purities were $\geq 99\%$.

combination with PF-04418948. Similar results were observed with PF-04418948 and CJ-042794 when misoprostol was used as the agonist (Fig. 4b). The EP₄-selective antagonist, L-161,982 (2 μ M), 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 PGE₂ inhibition were determined. The data demonstrated that increasing concentrations of the antagonist led to progressive rightward shifts in the concentration response curve to PGE₂ (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).

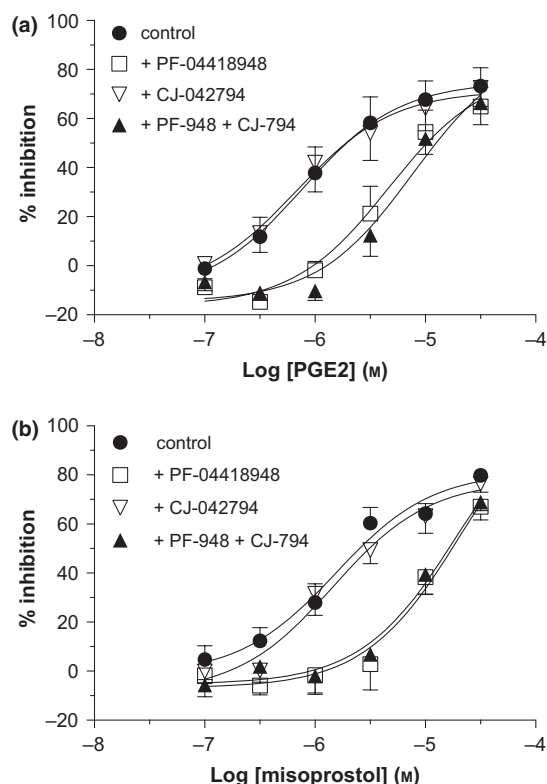


Fig. 4. Effect of EP receptor antagonists on (a) PGE₂ and (b) misoprostol. Mast cells were incubated (50 min) without or with the EP₂-selective antagonist PF-04418948 (300 nM) or the EP₄-selective antagonist CJ-042794 (1 μ M) or both antagonists and then without or with PGE₂ or misoprostol for 10 min before challenge with anti-IgE (2 μ g/mL) for 25 min. Values are expressed as the % inhibition of control histamine release which ranged from 25 ± 8 to $28 \pm 9\%$ for (a) and from 25 ± 3 to $26 \pm 4\%$ for (b). Values are means $\pm$ SEM for 4 and 5 experiments for (a) and (b) respectively.

An alternative EP₂-selective antagonist, PF-04852946, structurally distinct from PF-04418948, was also studied. PF-04852946 is about tenfold more potent than PF-04418948 at EP₂ 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 PGE₂ 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 EP₂ and EP₄ receptors, whereas LAD2 cells do not express either of these receptors, but do express message for EP₃ receptors (Fig. 6).

Additional studies were performed to determine whether PGE₂ had any inhibitory effects on histamine

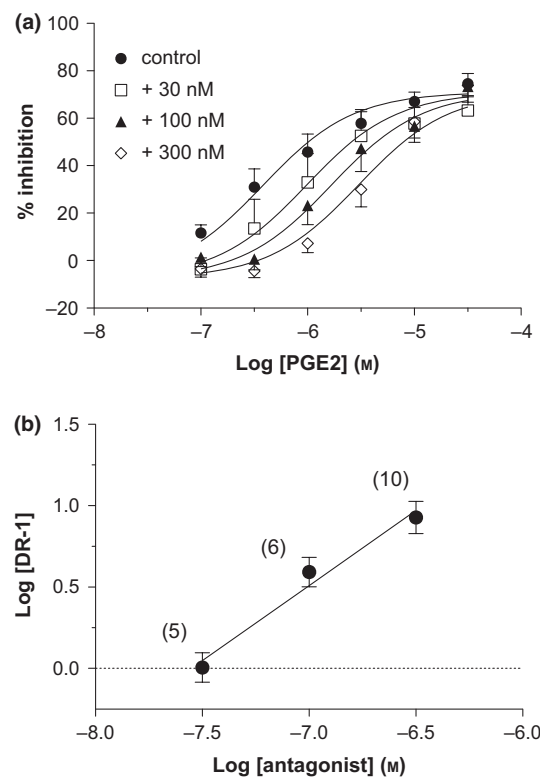


Fig. 5. Schild regression analysis. (a) Mast cells were incubated (50 min) without or with increasing concentrations of the EP₂-selective antagonist PF-04418948 and then without or with PGE₂ for 10 min before challenge with anti-IgE (2 μ g/mL) for 25 min. Values are expressed as the % inhibition of control histamine release which ranged from 25 ± 6 to $28 \pm 7\%$. Values are means $\pm$ 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 PGE₂. 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.

release from LAD2 cells. LAD2 cells were passively sensitized (20 h) with IgE (clone JW8, ~ 0.2 μ g/mL), the cells washed and then incubated (10 min) with PGE₂ (10^{-7} – 3×10^{-5} M) before challenge (25 min) with anti-IgE (2 μ g/mL). PGE₂ 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 PGE₂ on human lung mast cells. Previous studies of ours suggested that PGE₂ works through EP₂ receptors to stabilize mast cells [21]. However, this conclusion was drawn largely on the basis of studies utilizing antagonists of EP₂ and EP₄

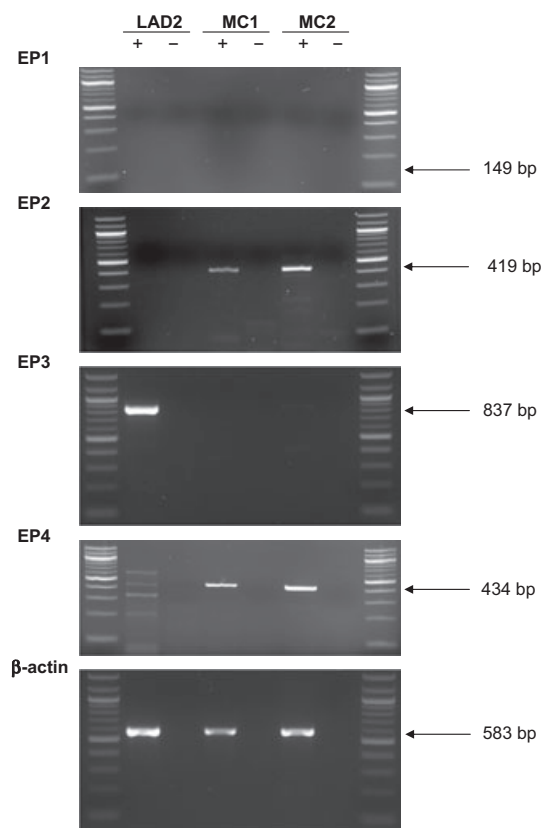


Fig. 6. EP receptor expression in mast cells and LAD2 cells. Isolated RNA was converted into cDNA by reverse transcriptase (+) and, as a control, this reaction step was also carried out in the absence of reverse transcriptase (-). Amplification of cDNA was performed using primers specific for each of the EP receptor subtypes and β -actin. Expression profiles for two mast cell preparations (MC1 and MC2) and LAD2 cells are shown. No EP₁ was detected in either cell type, but in separate experiments the presence of EP₁ 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 $\geq 97\%$.

receptors that lacked potency and selectivity. Moreover, recent studies in human bronchial smooth muscle suggest that the EP₄ receptor [17, 18], and not as previously thought the EP₂ receptor [16], mediates PGE₂-dependent bronchodilation. This has led to the suggestion that the EP₄ receptor could serve as a novel target for respiratory diseases [17, 18]. For these reasons, we have reappraised the actions of PGE₂ 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], PGE₂ 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 PGE₁

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 PGE₂ in mast cells. Misoprostol is a relatively non-selective agonist that is known to exert effects at EP₂, EP₃ and EP₄ receptors but not at EP₁ receptors. Moreover, it has been reported that misoprostol is about 30-fold less potent than PGE₂ at EP₃ and EP₄ receptors, but about sevenfold less potent than PGE₂ at EP₂ receptors [9]. The relative potencies of misoprostol and PGE₂ imply that mast cells are likely to express EP₂ receptors.

A response to butaprost is considered diagnostic for the functional expression of EP₂ receptors [8]. In this study, butaprost (free acid) inhibited histamine release, but was about eightfold less potent than PGE₂. Further studies were performed with ONO-AE1-259 which has been reported to be a potent and selective agonist at EP₂ 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 PGE₂. AH13205, which is also recognized as an EP₂ agonist [41], was also studied in this system and was found to be the least potent of all the EP agonists tested. Several EP₄ 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 EP₄ receptors, the concentration response curves were steep and maximal inhibition was greater than that seen with PGE₂.

In additional studies, the effects of PGE₂ on total cyclic-AMP levels in mast cells were evaluated. As the EP₂ receptor is coupled to adenylyl cyclase [10, 42], the demonstration that PGE₂, and misoprostol, elevated cyclic-AMP provides further evidence that the EP₂ receptor is expressed by mast cells. However, as the EP₄ receptor is also coupled to adenylyl cyclase [10] these data do not exclude the possible expression of EP₄ receptors by mast cells. It was of interest that EP₂-selective agonists, butaprost, ONO-AE1-259 and AH13205 raised cyclic-AMP in mast cells, whereas the EP₄-selective agonists, L-902,688 and TCS251, were ineffective. These data provide further evidence that PGE₂ acts through EP₂ receptors to attenuate mast cell responses.

Previous reports have indicated that cultured human mast cells, derived from either cord blood or peripheral blood, express EP₃ 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 PGE₂-mediated changes in calcium signalling were observed, arguing against the functional expression of either EP₃ or EP₁ receptors in these cells. These findings are also supported by our

previous studies showing that neither sulprostone (EP₃ agonist) nor 17-phenyl-trinor-PGE₂ (EP₁ 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 PGE₂ does not mediate effects through either EP₃ or EP₁ 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 EP₂ 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 EP₂ receptor antagonist, PF-04418948 [24], and this antagonist was used in this study. PF-04418948 was found to be an effective antagonist of the PGE₂ 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 PGE₂ and misoprostol are acting at the same receptor in human lung mast cells.

In further support of this reasoning, an alternative EP₂ antagonist was also studied, PF-04852946, which in other systems has been shown to be tenfold more potent at the EP₂ 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 PGE₂ and that this is likely to be the EP₂ receptor.

By contrast, the EP₄-selective antagonists, CJ-042794 and L-161,982, failed to reverse the PGE₂ inhibition in human mast cells. Attempts to unveil a potential EP₄-mediated effect of PGE₂ by incubating mast cells with an EP₂- and an EP₄-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 EP₂ receptor is the principal receptor through which PGE₂ 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 EP₄ agonists were effective inhibitors of IgE-dependent histamine release. This inhibitory effect of EP₄ agonists was not accompanied by increases in total cell cyclic-AMP, suggesting that if EP₄ 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 EP₄ agonist, L-902,688, were unaffected by either EP₄- or EP₂-selective antagonists. These data suggest that the effects of L-902,688 on mast cells are not due to interactions with either EP₄ or EP₂ 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 EP₂ and EP₄ receptors, but no evidence for EP₁ or EP₃ receptors. At a functional level, an overwhelming body of evidence indicates that PGE₂ acts through EP₂ receptors to modulate mast cell responses. Despite the apparent expression of EP₄ receptors there was little evidence for EP₄ involvement functionally. It is possible, however, that EP₄ receptors can contribute to the effects of PGE₂ 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 EP₃ receptors, and PGE₂ 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 PGE₂ acts through EP₂ receptors to stabilize human lung mast cells. This has been determined, principally, using potent and selective EP₂ 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 EP₂ 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 EP₄ 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 EP₄-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 EP₂ and EP₄ receptor antagonists utilised in these studies.

Figure S1. Effect of the EP₂ receptor antagonist, PF-04852946, on (a) PGE₂ and (b) misoprostol. Mast cells

were incubated (50 min) without or with PF-04852946 (30 nM) and then without or with PGE₂ or misoprostol for 10 min before challenge with anti-IgE (2 μ g/mL) for 25 min. Values are expressed as the % inhibition of control histamine release which were 23 ± 4 and $25 \pm 5\%$ for (a) and 22 ± 3 and $23 \pm 3\%$ for (b). Values are means $\pm$ SEM for 5 and 4 experiments for (a) and (b), respectively.