

RESEARCH PAPER

Ca²⁺ signals evoked by histamine H₁ receptors are attenuated by activation of prostaglandin EP₂ and EP₄ receptors in human aortic smooth muscle cells

Evangelia Pantazaka, Emily J A Taylor, William G Bernard and Colin W Taylor

Department of Pharmacology, University of Cambridge, Cambridge, UK

Correspondence

Colin W Taylor, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK. E-mail: cwt1000@cam.ac.uk

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BACKGROUND AND PURPOSE

Histamine and prostaglandin E₂ (PGE₂), directly and via their effects on other cells, regulate the behaviour of vascular smooth muscle (VSM), but their effects on human VSM are incompletely resolved.

EXPERIMENTAL APPROACH

The effects of PGE₂ on histamine-evoked changes in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) and adenylyl cyclase activity were measured in populations of cultured human aortic smooth muscle cells (ASMCs). Selective ligands of histamine and EP receptors were used to identify the receptors that mediate the responses.

KEY RESULTS

Histamine, via H₁ receptors, stimulates an increase in [Ca²⁺]_i that is entirely mediated by activation of inositol 1,4,5-trisphosphate receptors. Selective stimulation of EP₂ or EP₄ receptors attenuates histamine-evoked Ca²⁺ signals, but the effects of PGE₂ on both Ca²⁺ signals and AC activity are largely mediated by EP₂ receptors.

CONCLUSIONS AND IMPLICATIONS

Two important inflammatory mediators, histamine via H₁ receptors and PGE₂ acting largely via EP₂ receptors, exert opposing effects on [Ca²⁺]_i in human ASMCs.

Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; AC, adenylyl cyclase; AH6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; ASMC, aortic smooth muscle cell; BW 245C, (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; DDA, 2',5'-dideoxyadenosine; GW627368X, 2-[4-(4,9-diethoxy-3-oxo-1H-benzo[f]isoindol-2-yl)phenyl]-N-phenylsulfonylacetamide; HBS, HEPES-buffered saline; IP₃, inositol 1,4,5-trisphosphate; L902,688, 5-[(1E,3R)-4,4-difluoro-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2H-tetrazol-5R-yl)hexyl]-2-pyrrolidinone; NAADP, nicotinic acid adenine dinucleotide phosphate; pEC₅₀ (pIC₅₀), negative logarithm of the half-maximally effective (inhibitory) concentration; PLC, phospholipase C; SQ 22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; SQ/DDA, 1 mM SQ 22536 with 200 μM DDA (used to inhibit AC); SR, sarcoplasmic reticulum; *trans*-Ned-19, (1R,3S)-1-[3-[[4-(2-fluorophenyl)piperazin-1-yl]methyl]-4-methoxyphenyl]-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid; U73122, 1-[6-[[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione]; U73343, 1-[6-[[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione]; VSM, vascular smooth muscle

Introduction

Histamine released from mast cells and basophils initiates inflammatory responses, notably in the vasculature where its most immediate effects are dilatation of small vessels causing increased blood flow to sites of injury. These effects are mediated primarily by H₁ histamine receptors on endothelial cells, which stimulate Gq and thereby phospholipase C (PLC) (Hill *et al.*, 1997; Jones and Kearns, 2011). In larger vessels, including human arteries, H₁ receptors directly stimulate contraction (Toda, 1987) and proliferation of vascular smooth muscle (VSM) cells (Satoh *et al.*, 1994). There are, however, differences between species in the effects of histamine on the vasculature (Wang *et al.*, 1993, and references therein).

Inflammation plays a key role in the development of atherosclerosis (Hansson, 2005; Swedenborg *et al.*, 2011). Atherosclerotic lesions in human aorta are associated with enhanced local synthesis of histamine and increased expression of H₁ receptors (Takagishi *et al.*, 1995; Tanimoto *et al.*, 2006). Furthermore, in animal models, prevention of histamine synthesis (Toda, 1987; Sasaguri *et al.*, 2005) or competitive antagonists of H₁ receptors (Miyazawa *et al.*, 1998) reduce intimal thickening after vascular injury. These observations implicate H₁ receptors of VSM in both inflammatory responses and the development of atherosclerosis and restenosis.

Prostaglandin E₂ (PGE₂), the most abundant PG in humans, is another inflammatory mediator with widespread effects that include regulation of blood vessels (Norel, 2007). Human VSM synthesizes PGE₂ (Soler *et al.*, 2000), and its synthesis is much increased in diseased human aorta (Bayston *et al.*, 2003). PGE₂ is associated with development of atherosclerosis (Gomez-Hernandez *et al.*, 2006), and EP receptors are potential targets for its treatment (Yang *et al.*, 2011). The effects of PGE₂ are predominantly mediated by four classes of GPCRs. EP₁ receptors stimulate PLC via Gq, EP₂ and EP₄ receptors stimulate adenylyl cyclase (AC) via Gs, and most splice variants of EP₃ receptors regulate Gi (Norel, 2007; Sugimoto and Narumiya, 2007). There is evidence, albeit rather sparse in humans, suggesting that all four EP receptor subtypes are expressed in various VSM (Coleman *et al.*, 1994; see references in Breyer *et al.*, 2001) including EP₂ and EP₃ receptors in human aortic smooth muscle (Bayston *et al.*, 2003). Activation of EP₁, or more often EP₃, receptors causes contraction of various VSM (Jadhav *et al.*, 2004; Jones and Woodward, 2011; Kobayashi *et al.*, 2011) including human veins (Walch *et al.*, 2001) and arteries (Qian *et al.*, 1994; Jones *et al.*, 1997; references in Walch *et al.*, 2001). Stimulation of EP₂ or EP₄ receptors causes relaxation of many VSM (Jones *et al.*, 1997; Yang *et al.*, 2010), including human vessels (Qian *et al.*, 1994; Davis *et al.*, 2004; Foudi *et al.*, 2008). These responses are consistent with the common observation that PLC-coupled receptors generally cause Ca²⁺-mediated contraction of smooth muscle, including VSM, while those that stimulate AC cause relaxation (references in Roscioni *et al.*, 2010). The latter is widely thought to be mediated by cyclic AMP-dependent protein kinase, which via phosphorylation, can attenuate Ca²⁺ signalling and/or reduce the sensitivity of the contractile apparatus to Ca²⁺ (references in Roscioni *et al.*, 2010). Collectively, these observations suggest that PGE₂ and EP receptors play important roles in the normal physiology of

blood vessels, and they are implicated in various pathological states including atherosclerosis (Yang *et al.*, 2011).

The possibility that H₁ and/or EP receptors might be targeted for treatment of vascular diseases is attractive because H₁ receptor antagonists (Hill *et al.*, 1997) and PG analogues (Abramovitz *et al.*, 2000; Norel, 2007) are already established in clinical practice for other indications. Our choice of cultured VSM to investigate interactions between histamine and PGE₂ is vindicated by the experimental opportunities that would not be accessible in studies of tissues, and by evidence that development of atherosclerosis is associated with a phenotypic change of VSM from a contractile to a proliferating ('synthetic') phenotype (Orr *et al.*, 2010) similar to that of VSM in culture (House *et al.*, 2008).

In the present study, we demonstrate that in human aortic smooth muscle cells (ASMCs), activation of H₁ receptors by histamine stimulates an increase in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) entirely via activation of inositol 1,4,5-trisphosphate (IP₃) receptors. Activation of EP₂ or EP₄ receptors substantially attenuates the Ca²⁺ signals evoked by histamine, but EP₂ receptors are largely responsible for the inhibition of Ca²⁺ signals and stimulation of AC activity by PGE₂.

Methods

Culture of human ASMCs

Human ASMCs were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) or provided by Dr Trevor Littlewood (Division of Cardiovascular Medicine, University of Cambridge) (Boyle *et al.*, 2002). Ethical committee approval for the latter was obtained from the Addenbrooke's NHS Trust. All cells were isolated from patients who died of causes unrelated to the cardiovascular system. Further details of the cells and the specific experiments for which they were used are provided in Supporting Information Table S1. Cells were cultured in DMEM supplemented with GlutaMAX-I, heat-inactivated FBS (10%), penicillin (100 units·mL⁻¹) and streptomycin (0.1 mg·mL⁻¹) at 37°C in humidified air containing 5% CO₂. Cells from ATCC were first cultured according to the supplier's instructions (in ATCC vascular cell basal medium supplemented with ATCC VSM growth kit), and then as described earlier before use in experiments. Cells were used between passages 2 and 6, during which they retained an elongated shape and immunostained for α-smooth muscle actin (not shown).

Measurement of [Ca²⁺]_i in cell populations

Confluent cultures of ASMC grown in 96-well plates were loaded in HEPES-buffered saline (HBS) with fluo-4 by incubation (1 h, 20°C) with fluo-4-acetoxymethyl ester (4 μM, Invitrogen, Paisley, UK), probenecid (2.5 mM) and pluronic F127 (0.02%, v/v) (Govindan *et al.*, 2010). After a further incubation (30 min) in HBS supplemented with only probenecid, the medium was replaced with HBS and the cells were used for experiments. HBS had the following composition (mM): NaCl 135, KCl 5.9, MgCl₂ 1.2, CaCl₂ 1.5, glucose 11.5 and HEPES 11.6 (pH 7.3). Fluorescence (excitation at 485 nm, emission at 525 nm) was recorded during

appropriate additions using a FlexStation 3 fluorescence spectrometer (MDS Analytical Technologies, Wokingham, UK). Fluorescence signals (F) were calibrated to $[Ca^{2+}]_i$ from $[Ca^{2+}]_i = K_D(F - F_{min})/(F_{max} - F)$, where K_D is the equilibrium dissociation constant of fluo-4 for Ca^{2+} (345 nM) (Gee *et al.*, 2000), F_{min} is the fluorescence of Ca^{2+} -free indicator [recorded from cells treated with 0.05% Triton X-100 and 10 mM 1,2-bis(2-aminophenoxy)ethane- N,N,N' -tetraacetic acid (BAPTA) in Ca^{2+} -free HBS] and F_{max} is the fluorescence of Ca^{2+} -saturated indicator (cells treated with 0.05% Triton X-100 and 10 mM $CaCl_2$ in HBS). Ca^{2+} signals were recorded after addition of histamine alone or in the presence of additional drugs added 5 min before histamine. Further details are provided in the figure legends.

Measurement of AC activity

Two assays were used to determine AC activity in confluent cultures of ASMC. A HitHunter cAMP HS+ (DiscoverX, Birmingham, UK) enzyme complementation assay was used for most analyses. Briefly, confluent cultures of ASMC in 96-well plates were washed twice with HBS and incubated with appropriate stimuli (5 min). Reactions were stopped by aspirating the medium followed by addition of ice-cold ethanol (100 μ L). After 10 min, the ethanol was evaporated (60°C, 30 min). This heating step also inactivates an endogenous β -galactosidase-like activity that otherwise contributes to the final luminescence measurement. The cAMP contents of the extracts were then determined according to the manufacturer's instructions. Because the AC inhibitors used [1 mM SQ 22536 with 200 μ M DDA (SQ/DDA)] interfere with the HitHunter assay, analysis of their effects used a 3H -adenine-labelling assay in which cultures of ASMC in 24-well plates were incubated with [2,8- 3H] adenine (1 μ Ci·mL $^{-1}$, 1 mL per well) in DMEM for 2 h at 37°C in humidified air containing 5% CO $_2$. After washing twice with HBS, cells in HBS at 20°C were used for experiments. Incubations were terminated by aspirating the medium and then adding ice-cold trichloroacetic acid (5% v/v, 1 mL). After 30 min at 4°C, 3H -labelled adenine nucleotides were separated using sequential Dowex 50WX4-400 (Bio-Rad, Hemel Hempstead, UK) and alumina columns (Sigma-Aldrich, Poole, UK) (Salomon *et al.*, 1974).

Analyses of EP receptor expression by quantitative PCR (QPCR)

Confluent cultures of human ASMC in 24-well plates were lysed with Fastlane cDNA kit (500 μ L per well, Qiagen, Crawley, UK). QPCR reactions were conducted according to the manufacturer's instructions using Rotorgene SYBR green PCR kit (Qiagen) with an initial denaturation (95°C, 5 min) followed by 40 cycles of amplification (95°C for 5 s and 60°C for 10 s). Fluorescence was measured at the end of each cycle (Govindan *et al.*, 2010). Primers specific for EP $_2$ (Qiagen Quantitect Primer Assay, code: Hs_PTGER2_1_SG; Qiagen) or EP $_4$ receptors (Hs_PTGER4_2_SG) or, for calibration, primers for GAPDH (forward, ACCACAGTCCATGCCATCAC; reverse, TCCACCACCCTGTTGCTGTA) were used. The authenticity of each PCR product was confirmed by melting-curve analysis. Amplification efficiency (E) was calculated as 10^m , where m is the average increase in fluorescence for four cycles after the cycle threshold (C_T). Expression levels relative to the housekeeping product (GAPDH) were calculated from

$$\text{relative expression} = E^{-C_T^{EP}} / E^{-C_T^{GAPDH}}$$

Reactions were performed in duplicate with extracts from at least three different wells for each patient.

Statistical analysis

Concentration–effect relationships were individually fitted by non-linear curve fitting to Hill equations (GraphPad Prism version 5; GraphPad Software, La Jolla, CA, USA). Antagonist affinities (pK_D) were calculated from dose ratios. Two-tailed paired Student's t -test or one-way ANOVA with Bonferroni's *post hoc* test was used as appropriate, with $P < 0.05$ considered significant.

Materials

Cell culture materials, except FBS (Sigma), were from Invitrogen. Ionomycin, U73122 (1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), U73343 (1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione), SQ 22536 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine) and DDA (2',5'-dideoxyadenosine) were from Merck Chemicals Ltd. (Nottingham, UK). *trans*-Ned-19 [(1R,3S)-1-[3-[[4-(2-fluorophenyl)piperazin-1-yl]methyl]-4-methoxyphenyl]-2,3,4,9-tetrahydro-1H-pyrido[3,4-*b*]indole-3-carboxylic acid] was from Enzo Life Sciences (Exeter, UK). Thapsigargin was from Alomone Labs Ltd. (Jerusalem, Israel). Histamine dihydrochloride, cimetidine hydrochloride, mepyramine maleate, AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), BW 245C [(4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid], 2-APB (2-aminoethoxydiphenyl borate) and PGE $_2$ were from Sigma. Butaprost (free acid), GW627368X (2-[4-(4,9-diethoxy-3-oxo-1H-benzo[f]isoindol-2-yl)phenyl]-N-phenylsulfonylacetamide), latanoprost (free acid) and L902,688 (5-[(1E,3R)-4,4-difluoro-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2H-tetrazol-5R-yl)hexyl]-2-pyrrolidinone) were from Cayman Chemicals (Ann Arbor, MI, USA). Indomethacin and sulprostone were from R&D Systems (Minneapolis, MN, USA). Ryanodine was from AbCam (Cambridge, UK). [2,8- 3H] adenine (36 Ci·mmol $^{-1}$) was from PerkinElmer (Seer Green, Bucks, UK). All other reagents were from Sigma or sources specified in the relevant Methods section. Where dimethyl sulphoxide was used as a solvent (usually 0.01% v/v; 0.1% and 0.5% v/v in Figure 1F,E respectively), it was also included in controls. Key properties of the drugs used are provided in Supporting Information Table S2. The nomenclature of receptors and ligands follows Alexander *et al.* (2011).

Results

Histamine H_1 receptors evoke Ca^{2+} signals via IP $_3$ receptors in human ASMCs

Histamine evoked a concentration-dependent increase in $[Ca^{2+}]_i$ in populations of human ASMC. Removal of extracellular Ca^{2+} had no effect on the initial peak response to histamine, but it abolished the very small sustained increase in $[Ca^{2+}]_i$ (Figure 1A,B). The two phases of the Ca^{2+} signal, Ca^{2+} release from intracellular stores ($pEC_{50} = 6.56 \pm 0.09$, $n = 4$)

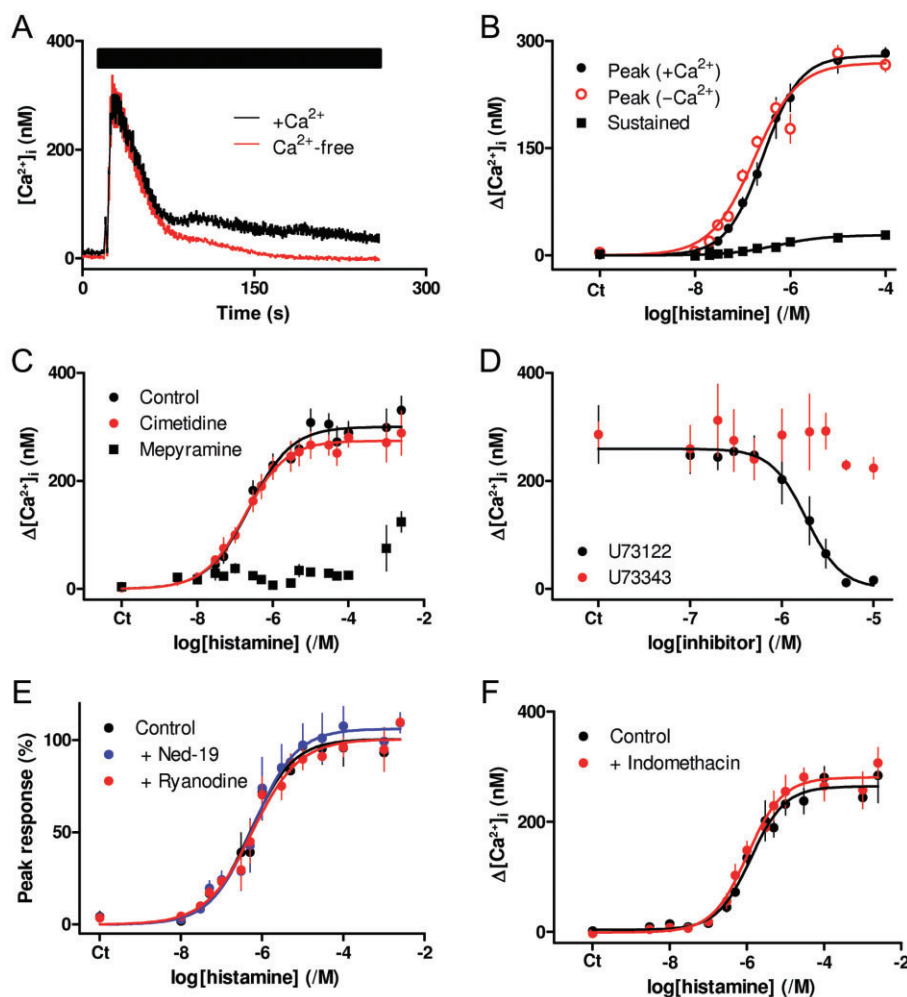


Figure 1

Histamine H₁ receptors stimulate an increase in [Ca²⁺]_i via IP₃. (A) Histamine (100 μM, bar) stimulates an increase in [Ca²⁺]_i in populations of ASMC incubated in HBS or Ca²⁺-free HBS. Results show means ± SEM from three wells on a single 96-well plate and are typical of results from four independent plates. (B) Concentration-dependent effects of histamine on the peak [Ca²⁺]_i in the presence or absence of extracellular Ca²⁺, and on the sustained Ca²⁺ entry (measured ~230 s after histamine addition). Results are means ± SEM from four independent plates, with one to three wells on each plate. (C) Effect of mepyramine (0.5 μM) and cimetidine (50 μM), each added 5 min before histamine, on histamine-evoked Ca²⁺ signals. Results are means ± SEM from nine independent plates, with one to three wells on each plate. (D) Effects of the indicated concentrations of U73122 and U73343 (added 5 min before histamine) on the peak increase in [Ca²⁺]_i evoked by histamine (100 μM). Results are means ± SEM from three independent plates, with two wells on each plate. (E) Effects of *trans*-Ned-19 (1 μM) and ryanodine (100 μM), each added 5 min before histamine, on the peak increase in [Ca²⁺]_i evoked by histamine. Results (percentage of the maximal response) are means ± SEM from three independent plates with one to three wells on each plate. (F) Effect of indomethacin (10 μM, added 5 min before histamine) on the peak Ca²⁺ signals evoked by histamine. Results are means ± SEM from four independent plates, each with three wells. (B–F) Ct denotes control.

and Ca²⁺ entry (pEC₅₀ = 6.12 ± 0.30, *n* = 4), were similarly sensitive to histamine. Responses to histamine were unaffected by cimetidine (50 μM, 5 min), a selective antagonist of H₂ histamine receptors, but mepyramine (0.5 μM, 5 min), a competitive antagonist of H₁ histamine receptors (Alexander *et al.*, 2011), shifted the concentration–effect relationship to ~26 000-fold higher concentrations of histamine, suggesting a pK_D for mepyramine of ~10.7 (Figure 1C). Pre-equilibration with mepyramine, which dissociates slowly from H₁ histamine receptors, and the need to measure acute responses to histamine probably exaggerate the effect the antagonist would have at equilibrium, but the results are consistent with

histamine, evoking Ca²⁺ signals via a receptor with high affinity for mepyramine and undetectable affinity for cimetidine. U73122, an inhibitor of PLC (Bleasdale *et al.*, 1990), caused a concentration-dependent inhibition (pIC₅₀ = 5.76 ± 0.09, *n* = 3) of the Ca²⁺ signals evoked by histamine, while its inactive analogue, U73343 (≤10 μM), had no effect (Figure 1D).

Similar results, although with slightly different sensitivities to histamine and peak Ca²⁺ signals, were obtained with ASMC isolated from different patients (Supporting Information Figure S1). These differences, presumably arising from different levels of expression of H₁ receptors or downstream

signalling proteins, highlight the need for paired comparisons of experimental manipulations in subsequent analyses of human ASMC. It also justifies our presentation of some results as percentages of matched control responses.

There are no membrane-permeant selective antagonists of IP₃ receptors, although 2-APB has often been used (Taylor and Tovey, 2010). 2-APB caused a concentration-dependent inhibition of the Ca²⁺ signals evoked by either maximally or submaximally effective concentrations of histamine (Supporting Information Figure S2A–D). However, in keeping with published results (Peppiatt *et al.*, 2003), similar concentrations of 2-APB also caused a substantial loss of Ca²⁺ from the intracellular stores (Supporting Information Figure S2E,F). While the slightly greater sensitivity of the histamine responses to 2-APB is consistent with the expected involvement of IP₃ receptors, the results highlight the limitations of 2-APB as a useful antagonist of IP₃ receptors.

Ryanodine receptors and the intracellular Ca²⁺ channels that are activated by nicotinic acid adenine dinucleotide phosphate (NAADP), probably two-pore channels (Calcraft *et al.*, 2009), can also mediate Ca²⁺ release from intracellular stores, including those of VSM (Boittin *et al.*, 2002; Tugba Durlu-Kandilci *et al.*, 2010). However, caffeine (10 mM), which activates ryanodine receptors, had no effect on [Ca²⁺]_i (Supporting Information Figure S3A), and the responses to histamine were unaffected by concentrations of either *trans*-Ned-19 (1 µM, 5 min) that block responses to NAADP (Naylor *et al.*, 2009; Brailoiu *et al.*, 2010) or of ryanodine (100 µM, 5 min) that block ryanodine receptors (Zheng *et al.*, 2005) (Figure 1E).

These results demonstrate that activation of H₁ receptors by histamine stimulates Ca²⁺ release via IP₃ receptors in human ASMC, and that neither ryanodine receptors nor two-pore channels contribute to the response. Our results are consistent with widespread expression of H₁ histamine receptors in most smooth muscles (Hill *et al.*, 1997) and with evidence that histamine, via H₁ receptors, stimulates PLC and an increase in [Ca²⁺]_i in human ASMC (Sato *et al.*, 1994). In light of subsequent evidence demonstrating effects of PGE₂ and cAMP on Ca²⁺ signals, we considered the possibility that histamine might influence Ca²⁺ signals via activation of endogenous H₂ receptors or stimulate production of endogenous prostanoids. However, neither cimetidine, a selective antagonist of H₂ receptors (Figure 1C), nor indomethacin, an inhibitor of cyclooxygenases (Figure 1F), had any effect on histamine-evoked Ca²⁺ signals in human ASMC.

PGE₂ inhibits histamine-evoked Ca²⁺ signals

PGE₂ alone had no effect on [Ca²⁺]_i (Supporting Information Figure S3B), but the Ca²⁺ signals evoked by histamine (100 µM) were attenuated by PGE₂ (10 µM, 5 min) (Figure 2A). PGE₂ decreased the sensitivity of ASMC to histamine (pEC₅₀ = 6.32 ± 0.10 and 5.74 ± 0.12, *n* = 7, in control and PGE₂-treated cells respectively) and it reduced the maximal response by 51 ± 2% (Figure 2B). PGE₂ had similar effects on both phases of the Ca²⁺ signal: Ca²⁺ release and the small Ca²⁺ entry (Figure 2C). The effects of PGE₂ on the peak increase in [Ca²⁺]_i evoked by histamine (3 µM) were concentration dependent (pIC₅₀ = 8.99 ± 0.10, *n* = 15; Figure 2D). Similar results, although again with some variation in absolute sensitivities, were obtained from different patients (Sup-

porting Information Figure S1 and Table S3). PGE₂ (10 µM) did not affect the Ca²⁺ content of the intracellular stores whether assessed by addition, in Ca²⁺-free HBS, of thapsigargin or cyclopiazonic acid to inhibit the Ca²⁺ pump (SERCA) of the sarcoplasmic reticulum (SR), or of ionomycin to release Ca²⁺ directly (Figure 2E). These results demonstrate that PGE₂ causes a concentration-dependent inhibition of the Ca²⁺ signals evoked by activation of H₁ histamine receptors without affecting the Ca²⁺ content of the SR.

Activation of EP₂ or EP₄ receptors inhibits histamine-evoked Ca²⁺ signals

PGE₂ activates many prostanoid receptors, with EP_{1–4}, DP₁ and FP receptors being the most sensitive (Abramovitz *et al.*, 2000; Alexander *et al.*, 2011). EP₁ receptors cannot mediate inhibition of histamine-evoked Ca²⁺ signals because their coupling to G_q would be expected to increase [Ca²⁺]_i. Sulprostone binds selectively to EP₃ receptors, with an affinity similar to that of PGE₂. BW 245C and latanoprost are selective high-affinity agonists of DP₁ and FP receptors respectively (Abramovitz *et al.*, 2000) (Supporting Information Table S2). Sulprostone (1 µM), BW 245C (10 nM) and latanoprost (100 nM) had no effect on histamine-evoked Ca²⁺ signals (Supporting Information Figure S3C), suggesting that neither EP₃, DP₁ nor FP receptors contribute to the inhibition of Ca²⁺ signals by PGE₂. Subsequent experiments therefore assess the contributions of EP₂ and EP₄ receptors, both of which are expressed in human VSM (Qian *et al.*, 1994; Bayston *et al.*, 2003; Davis *et al.*, 2004; Foudi *et al.*, 2008).

QPCR analysis of ASMC from two patients established that transcripts for EP₂ and EP₄ receptors were expressed at similar levels. In patients a and d (Supporting Information Table S1), 48 ± 2% and 59 ± 1% of the transcripts were for the EP₂ receptor. Although we have not directly assessed protein expression, the results are consistent with comparable levels of expression of EP₂ and EP₄ receptors in human ASMC.

Butaprost, a selective agonist of EP₂ receptors (Sugimoto and Narumiya, 2007; Alexander *et al.*, 2011), caused a concentration-dependent inhibition of the Ca²⁺ signals evoked by 3 µM histamine (pIC₅₀ = 8.22 ± 0.05, *n* = 3) (Figure 3A, Table 1). AH6809 is a poorly selective low-affinity antagonist of EP₂ receptors (pK_D = 5.9), but it does not interact with EP₄ receptors (Supporting Information Table S4). The response to butaprost was competitively antagonized by AH6809 (30 µM, ΔpIC₅₀ = 1.12 ± 0.15, where ΔpIC₅₀ = pIC₅₀^{control} – pIC₅₀^{+antagonist}), but insensitive to GW627368X (1 µM, ΔpIC₅₀ = –0.21 ± 0.14), an antagonist of EP₄ receptors (Wilson *et al.*, 2006) (Figure 3A, Table 1). These results show that butaprost inhibits histamine-evoked Ca²⁺ signals via a receptor with an affinity (pK_D) for AH6809 of 5.6. This confirms that the inhibition is mediated by EP₂ receptors (Supporting Information Table S4).

L902,688 is a selective agonist of EP₄ receptors with slightly greater affinity than PGE₂ (Young *et al.*, 2004) (Supporting Information Table S4). L902,688 also caused a concentration-dependent inhibition of histamine-evoked Ca²⁺ signals (pIC₅₀ = 9.52 ± 0.25, *n* = 3) (Figure 3B, Table 1). This inhibition was insensitive to AH6809 (30 µM, ΔpIC₅₀ = –0.01 ± 0.27), but competitively inhibited by GW627368X (1 µM, ΔpIC₅₀ = 2.20 ± 0.28). The latter suggests that L902,688 acts via a receptor with an affinity (pK_D) for GW627368X of

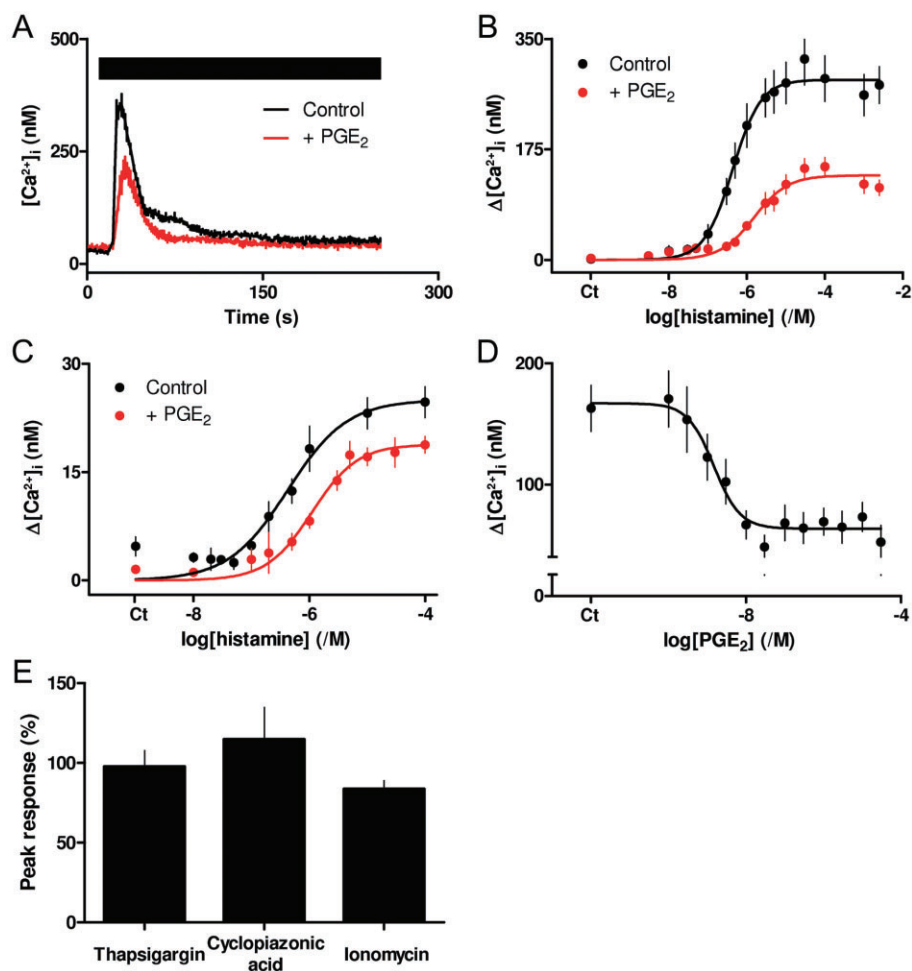


Figure 2

PGE₂ inhibits histamine-evoked Ca²⁺ release. (A) Ca²⁺ signals evoked by histamine (100 μM, bar) alone or with PGE₂ (10 μM, added 5 min before and then with histamine). Results, means ± SEM from three wells on a single plate, are typical of results from four independent plates. (B) Effect of PGE₂ (10 μM) on the peak Ca²⁺ signals evoked by the indicated concentrations of histamine. Results are means ± SEM from seven independent plates, each with one to three wells. (C) Effect of PGE₂ on the sustained Ca²⁺ signals evoked by histamine. Results are means ± SEM from 11 independent plates, each with one to three wells. (D) Effect of the indicated concentrations of PGE₂ (added 5 min before histamine) on the peak increase in [Ca²⁺]_i evoked by histamine (3 μM). Results are means ± SEM from 15 independent plates, with one to three wells analysed from each. (B–D) Ct denotes control. Similar results from ASMC isolated from different patients are shown in Supporting Information Figure S1. (E) Effects of pretreatment with PGE₂ (10 μM, 5 min) on the peak Ca²⁺ signals evoked by subsequent addition of thapsigargin (1 μM), cyclopiazonic acid (10 μM) or ionomycin (1 μM) to ASMC in Ca²⁺-free HBS. Results (as percentages of the responses obtained without PGE₂) are means ± SEM from three independent plates, with seven wells analysed on each.

8.2, consistent with it being an EP₄ receptor (Supporting Information Table S4). We conclude that selective activation of either EP₂ or EP₄ receptors in human ASMC inhibits histamine-evoked Ca²⁺ release. For butaprost, the sensitivity of the functional response (pIC₅₀ = 8.22) and the published binding affinity (pK_D = 7.04) differ by more than 10-fold, whereas they are more similar for L902,688 (9.5 and 9.4 respectively). This suggests that for inhibition of histamine-evoked Ca²⁺ signals, there is a greater receptor reserve for EP₂, than for EP₄, receptors.

PGE₂ inhibits histamine-evoked Ca²⁺ signals largely via EP₂ receptors

Maximally effective concentrations of PGE₂, butaprost or L902,688, caused similar inhibition of histamine-evoked Ca²⁺

signals, and no combination of the three stimuli caused any greater inhibition than a single stimulus (Figure 4A,B). This suggests that the three stimuli converge to cause inhibition via a common pathway. Subsequent experiments resolve which of the two receptors (EP₂ or EP₄) mediates the response to the endogenous stimulus, PGE₂.

Inhibition of histamine-evoked Ca²⁺ signals by PGE₂ (pIC₅₀ = 9.27 ± 0.12, *n* = 4) was antagonized by AH6809 (30 μM, ΔpIC₅₀ = 0.62 ± 0.13), but to a lesser extent than the response to butaprost (ΔpIC₅₀ = 1.12 ± 0.15). GW627368X (1 μM) also inhibited the responses to PGE₂ (ΔpIC₅₀ = 0.24 ± 0.08), but to a much lesser degree than the response to L902,688 (ΔpIC₅₀ = 2.20 ± 0.28) (Figure 4C, Table 1). These results suggest that both EP₂ and EP₄ receptors contribute to the PGE₂-mediated inhibition of histamine-evoked Ca²⁺

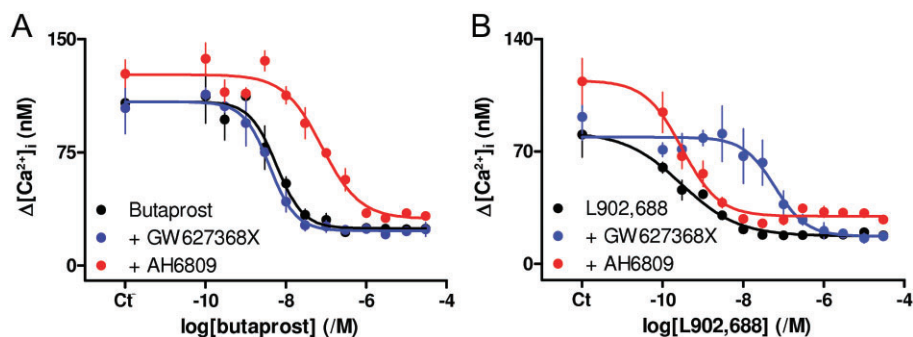


Figure 3

Activation of EP₂ or EP₄ receptors inhibits histamine-evoked Ca²⁺ signals. (A, B) Effect of butaprost (A, 5 min) or L902,688 (B, 5 min) on the Ca²⁺ signals evoked by histamine (3 μ M) alone or in the presence of AH6809 (30 μ M, 5 min) or GW627368X (1 μ M, 5 min). Results (means $\pm$ SEM from three independent plates, with two wells analysed from each) show amplitudes of the peak changes in [Ca²⁺]_i evoked by histamine. Ct denotes control. Summary results are shown in Table 1.

Table 1

Effects of PGE₂ and selective ligands of EP₂ and EP₄ receptors on histamine-evoked Ca²⁺ signals and cAMP accumulation

		[Ca ²⁺] _i , pIC ₅₀ (/M)		cAMP, pEC ₅₀ (/M)
	Control	AH6809	GW627368X	Control
PGE ₂	9.27 $\pm$ 0.12 (4)	8.65 $\pm$ 0.07* (4)	9.03 $\pm$ 0.13 (4)	6.62 $\pm$ 0.10 (4)
Butaprost	8.22 $\pm$ 0.05 (3)	7.10 $\pm$ 0.19* (3)	8.43 $\pm$ 0.17 (3)	5.68 $\pm$ 0.09 (4)
L902,688	9.52 $\pm$ 0.25 (3)	9.53 $\pm$ 0.05 (3)	7.31 $\pm$ 0.21* (3)	7.76 $\pm$ 0.11 (3)

Concentration-dependent effects of the indicated agonists of EP receptors on the increase in [Ca²⁺]_i evoked by histamine (3 μ M) (pIC₅₀) or cAMP accumulation (pEC₅₀) are shown alone or in the presence of antagonists of EP₂ (AH6809, 30 μ M) or EP₄ (GW627368X, 1 μ M) receptors. Measurements of cAMP accumulation were performed using the HitHunter HS+ assay. Results are means $\pm$ SEM, with the number of independent experiments shown in parentheses.

**P* < 0.05 relative to control.

signals, but the magnitudes of the disparities between the effects of the competitive antagonists on responses to PGE₂ and those evoked by the subtype-selective agonists (Figure 4D) suggest that EP₂ receptors mediate most responses to PGE₂. EP₄ receptors bind PGE₂ with approximately sixfold greater affinity than EP₂ receptors (Abramovitz *et al.*, 2000). The predominant role of the latter must, therefore, be due to their greater expression or more effective coupling to downstream signalling, consistent with our suggestion that only EP₂ receptors have a receptor reserve. Our evidence that transcripts for EP₂ and EP₄ receptors are expressed at similar levels would be consistent with more effective coupling of EP₂ receptors.

EP₂ and EP₄ receptors differ in their abilities to stimulate AC

Because EP₂ and EP₄ receptors share an ability to activate AC via the G protein Gs (Alexander *et al.*, 2011), we examined the effects of their activation on cAMP accumulation in human ASMC. PGE₂ evoked a concentration-dependent accumulation of cAMP whether assessed using ³H-adenine labelling (pEC₅₀ = 6.67 $\pm$ 0.06, *n* = 3) or the HitHunter assay (pEC₅₀ = 6.62 $\pm$ 0.10, *n* = 4) (Figure 5). This stimulation of

cAMP accumulation was reduced by 83 $\pm$ 1% (*n* = 3) in the presence of inhibitors of AC, SQ 22536 (1 mM) and DDA (200 μ M) (hereafter described as SQ/DDA) (Figure 5B). We note that as with histamine-evoked Ca²⁺ signals, there was a variability between patients and cell passages in the absolute amounts of cAMP produced in response to PGE₂, again dictating the need for paired comparisons of treatments (Table 1). These results are consistent with an earlier report in which PGE₂, but not histamine, evoked an increase in cAMP in human ASMC (Satoh *et al.*, 1994). Butaprost also stimulated AC activity (pEC₅₀ = 5.68 $\pm$ 0.09, *n* = 4; Figure 5A, Table 1). The similar maximal effects of PGE₂ and butaprost, and the ~10-fold higher EC₅₀ of the latter, are consistent with evidence that butaprost is a full agonist of human EP₂ receptors with ~20-fold lower affinity than PGE₂ (Narumiya *et al.*, 1999; Abramovitz *et al.*, 2000) (Supporting Information Table S4).

L902,688, which has approximately twofold greater affinity than PGE₂ for EP₄ receptors (Supporting Information Table S4), stimulated a concentration-dependent (pEC₅₀ = 7.76 $\pm$ 0.11) accumulation of cAMP that was only 32 $\pm$ 2% (*n* = 3) of that evoked by a maximally effective concentration of PGE₂ (Figure 5A). The lesser maximal response to L902,688

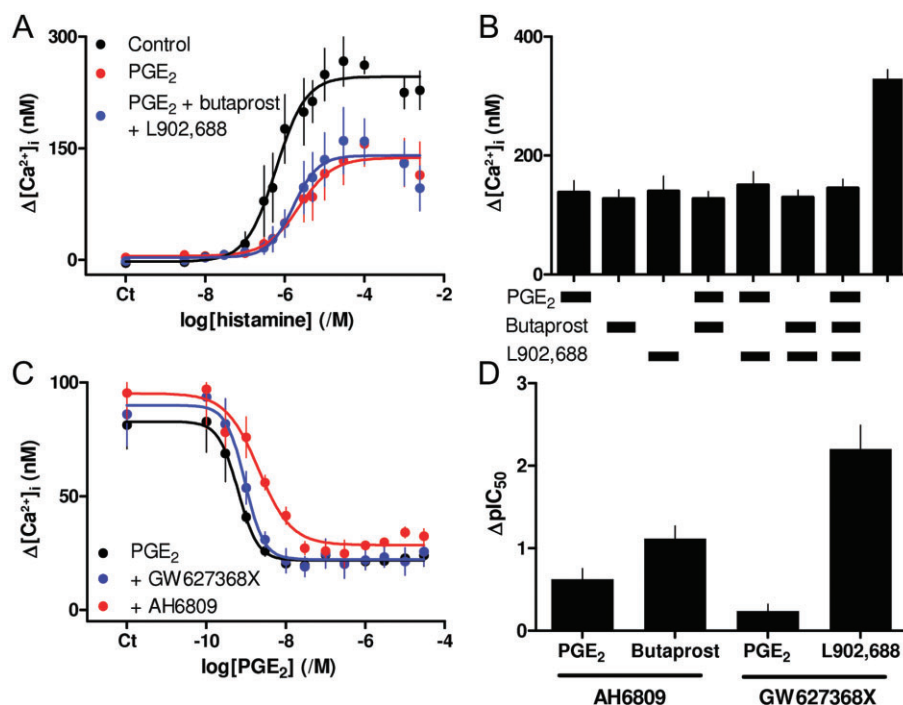


Figure 4

PGE₂ inhibits histamine-evoked Ca²⁺ signals largely via EP₂ receptors. (A) Effect of PGE₂ (10 μM) alone or in combination with butaprost and L902,688 (10 μM each) on the peak Ca²⁺ signals evoked by the indicated concentrations of histamine. Results are means ± SEM from three independent plates, each with one to two wells. (B) Peak Ca²⁺ signals evoked by histamine (3 μM) alone or with (5 min) PGE₂ (10 μM), butaprost (100 μM), L902,688 (10 μM) or the indicated combinations. Results are means ± SEM from 11 to 14 wells. (C) Effect of PGE₂ (5 min) on the peak Ca²⁺ signals evoked by histamine (3 μM) alone or in the presence of AH6809 (30 μM, 5 min) or GW627368X (1 μM, 5 min). Results are means ± SEM from four independent plates, each with one to two wells. (A, C) Ct denotes control. (D) ΔpIC₅₀ values for PGE₂, butaprost and L902,688 and the antagonists of EP₂ (AH6809, 30 μM) or EP₄ (GW627368X, 1 μM) receptors. Results are means ± SEM from three to four independent experiments.

and greater sensitivity relative to PGE₂ (Figure 5A) suggest that EP₄ receptors probably mediate the effect of L902,688 on AC activity (Supporting Information Table S4). We suggest that both EP₂ and EP₄ receptors stimulate AC in human ASMC, but the former is either more abundant or more effectively coupled to AC, and largely mediates the effects of PGE₂. More effective coupling of EP₂ receptors would be consistent with our analysis of transcripts for EP₂ and EP₄ receptors, and with analyses showing that EP₂ receptors evoke greater stimulation of AC than do EP₄ receptors when each is heterologously expressed at a similar level (Fujino *et al.*, 2002).

Discussion

Activation of histamine H₁ receptors evokes an increase in [Ca²⁺]_i in human ASMC that results from Ca²⁺ release via IP₃ receptors, followed by a small sustained response mediated by Ca²⁺ entry, most likely via a store-operated Ca²⁺ entry pathway (Figure 1). Such biphasic Ca²⁺ signals are typical of those evoked by PLC-coupled receptors in VSM (Berridge, 2008) and other tissues (Putney, 1997). PGE₂ is another inflammatory mediator with widespread actions in the vasculature (Norel, 2007). The receptors through which PGE₂ regulates human ASMC have not hitherto been defined,

although both EP₂ and EP₄ receptors mediate relaxation and/or attenuation of Ca²⁺ signalling in other human smooth muscles (Baxter *et al.*, 1995; Jones *et al.*, 1997; Benyahia *et al.*, 2012).

We have shown that in human ASMC, PGE₂ attenuates histamine-evoked Ca²⁺ signals without affecting the Ca²⁺ content of the intracellular stores (Figure 2). This inhibition is mediated by cAMP (Pantazaka *et al.*, unpubl. obs.). Inhibition of histamine-evoked Ca²⁺ signals is mimicked by selective activation of either EP₂ or EP₄ receptors (Figures 3 and 4A–C), but results with selective antagonists suggest that EP₂ receptors predominantly mediate the effect of PGE₂ on [Ca²⁺]_i (Figure 4D). This is consistent with the relationships between pIC₅₀ and published K_D values for the subtype-selective agonists suggesting a greater receptor reserve for EP₂ receptors. It also aligns with evidence that both EP₂ and EP₄ receptors stimulate AC activity, but the maximal response is much larger for EP₂ receptors (Figure 5A). The predominant role of EP₂, relative to EP₄, receptors, despite the lower K_D of the latter (Supporting Information Table S4), could arise from higher levels of expression of EP₂ receptors in human ASMC or their more effective coupling to stimulation of AC (Fujino *et al.*, 2002). The latter seems more likely because transcripts for EP₂ and EP₄ receptors are expressed at similar levels in human ASMC. There is some evidence that functional EP

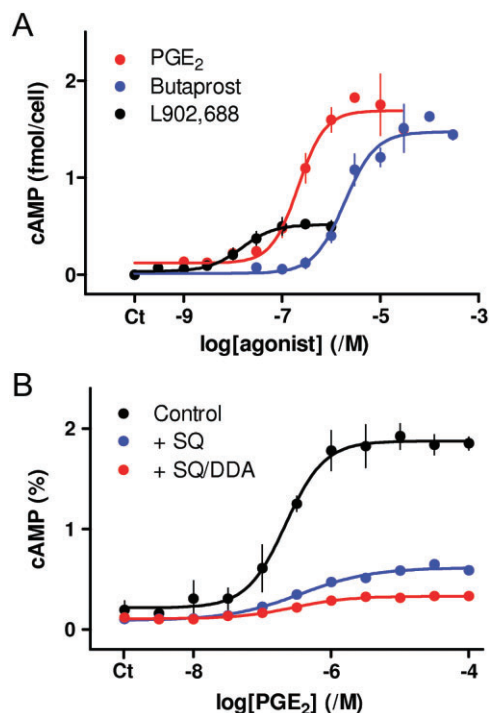


Figure 5

PGE₂ stimulates AC predominantly via EP₂ receptors. (A) Effects of the indicated concentrations of PGE₂, butaprost and L902,688 (5 min) on cAMP formation measured using a HitHunter assay. (B) Effect of PGE₂ (5 min) on cAMP accumulation (determined using ³H-adenine labelling, and expressed as percentages of ³H-ATP + ³H-ADP) alone or after treatment with SQ 22536 (1 mM SQ) alone ($n = 1$) or SQ 22536 with DDA (200 μ M) ($n = 3$). Both inhibitors were added 20 min before and then during stimulation with PGE₂. Results (except for SQ alone in panel B) are means $\pm$ SEM from three to four independent plates. Ct denotes control.

receptors, including EP₂ and EP₄ receptors, are expressed in both the plasma membrane and the nuclear envelope of some cells (Zhu *et al.*, 2006). We have not assessed whether this contributes to the ability of PGE₂ to inhibit histamine-evoked Ca²⁺ signals.

Our results demonstrate that two important inflammatory mediators, histamine and PGE₂, acting via receptors that are targets of existing clinically approved drugs, interact to control [Ca²⁺]_i in human ASMC. EP₂, EP₄ and histamine H₁ receptors might thereby provide effective therapeutic targets for treatment of atherosclerosis.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Inhibition of histamine-evoked Ca²⁺ signals by PGE₂ in ASMC from different patients. (A–C) Peak Ca²⁺ signals evoked by the indicated concentrations of histamine alone or with PGE₂ (10 µM added 5 min before histamine). Results are from three different patients (codes in Supporting Information Table S1) and show means ± SEM from five (A), 15 (B) and 11 (C) independent experiments. (D). Effect of the indicated concentrations of PGE₂ (added 5 min before histamine) on the pEC₅₀ for histamine-evoked peak Ca²⁺ signals. Ct denotes control.

Figure S2 Inhibition of histamine-evoked Ca²⁺ signals and depletion of intracellular Ca²⁺ stores by 2-APB. (A, C) Effect of 2-APB (added 5 min before histamine) on the Ca²⁺ signals evoked by submaximal (3 µM) (A) or maximal (1 mM) (C) concentrations of histamine in Ca²⁺-free HBS. (E) Similar analyses of the effects of 2-APB on the Ca²⁺ content of the intracellular stores assessed by addition of ionomycin (1 µM). The code shown in panel E applies to all three panels (B, D, F). Summary results show peak Ca²⁺ signals evoked by histamine (B, D) or ionomycin (F) after the indicated treatments with 2-APB. Results (A–F) are means ± SEM from three independent plates, with two wells analysed from each.

Figure S3 Caffeine and PGE₂ do not directly affect [Ca²⁺]_i, and neither EP₃, DP₁ nor FP receptors contribute to inhibition of Ca²⁺ signals by PGE₂. (A) Populations of ASMC in HBS were stimulated with caffeine (A, 10 mM) and then histamine (3 µM). (B) In similar experiments, ASMCs were stimulated with histamine alone (3 µM) or after pretreatment with PGE₂ (10 µM) as shown. The trace shows that although PGE₂ inhibits histamine-evoked Ca²⁺ signals, it does not itself affect [Ca²⁺]_i. Results (A and B) are means ± SEM from four wells on a single 96-well plate and are typical of results from three independent experiments. (C) The peak increase in [Ca²⁺]_i evoked by histamine (3 µM) is shown for histamine alone or in the presence of PGE₂ (100 nM) or agonists selective for EP₃ receptors (sulprostone, 1 µM), DP₁ receptors (BW 245C, 10 nM) or FP receptors (latanoprost, 100 nM) (each added 5 min before histamine). Results are means ± SEM from 8–29 wells.

Table S1 Sources of the human ASMC used.

Table S2 Key properties of the drugs used.

Table S3 Attenuation of histamine-evoked Ca²⁺ signals in human ASMC from different patients.

Table S4 Reported affinities (pK_D) of drugs used for analysis of EP₂ and EP₄ receptors.