British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01807.x
www.brjpharmacol.org

RESEARCH PAPER

The GPCR OGR1 (GPR68) mediates diverse signalling and contraction of airway smooth muscle in response to small reductions in extracellular pH

H Saxena[1], DA Deshpande[1], BC Tiegs[1], H Yan[1], RJ Battafarano[2], WM Burrows[2], G Damera[3], RA Panettieri[3], TD DuBose Jr[4], SS An[5] and RB Penn[1]

[1]*Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD, USA,* [2]*Division of Thoracic Surgery, Department of Surgery, University of Maryland School of Medicine, Baltimore, MD, USA,* [3]*Airways Biology Initiative, Pulmonary, Allergy and Critical Care Division, Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA,* [4]*Department of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, NC, USA, and* [5]*Program in Respiratory Biology and Lung Disease, Department of Environmental Health Sciences, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, MD, USA*

Correspondence
Raymond B Penn, Department of Medicine, University of Maryland School of Medicine, 20 Penn Street HSF-II Room 147, Baltimore, MD 21201-1075, USA. E-mail: rpenn@medicine.umaryland.edu

Keywords
airway smooth muscle; asthma; bronchoconstriction; calcium; cAMP-dependent protein kinase; contraction; GPR68; G protein-coupled receptor; inflammation; OGR1

Received
18 August 2011
Revised
31 October 2011
Accepted
16 November 2011

BACKGROUND AND PURPOSE

Previous studies have linked a reduction in pH in airway, caused by either environmental factors, microaspiration of gastric acid or inflammation, with airway smooth muscle (ASM) contraction and increased airway resistance. Neural mechanisms have been shown to mediate airway contraction in response to reductions in airway pH to < 6.5; whether reduced extracellular pH (pHo) has direct effects on ASM is unknown.

EXPERIMENTAL APPROACH

Intracellular signalling events stimulated by reduced pHo in human cultured ASM cells were examined by immunoblotting, phosphoinositide hydrolysis and calcium mobilization assays. ASM cell contractile state was examined using magnetic twisting cytometry. The expression of putative proton-sensing GPCRs in ASM was assessed by real-time PCR. The role of ovarian cancer G protein-coupled receptor 1 (OGR1 or GPR68) in acid-induced ASM signalling and contraction was assessed in cultures subjected to siRNA-mediated OGR1 knockdown.

KEY RESULTS

ASM cells responded to incremental reductions in pHo (from pH 8.0 to pH 6.8) by activating multiple signalling pathways, involving p42/p44, PKB, PKA and calcium mobilization. Coincidently, ASM cells contracted in response to decreased pHo with similar 'dose'-dependence. Real-time PCR suggested OGR1 was the only proton-sensing GPCR expressed in ASM cells. Both acid-induced signalling (with the exception of PKB activation) and contraction were significantly attenuated by knockdown of OGR1.

CONCLUSIONS AND IMPLICATIONS

These studies reveal OGR1 to be a physiologically relevant GPCR in ASM cells, capable of pleiotropic signalling and mediating contraction in response to small reductions in extracellular pH. Accordingly, ASM OGR1 may contribute to asthma pathology and represent a therapeutic target in obstructive lung diseases.

Abbreviations

ASM, airway smooth muscle; BK, bradykinin; EBC, exhaled breath condensate; OGR1(GPR68), ovarian cancer G protein-coupled receptor 1; PDGF, platelet derived growth factor; pHo, extracellular pH; PI, phosphoinositide; PKA, cAMP-dependent protein kinase; TDAG8, T cell death-associated gene 8; VASP, vasodilator-stimulated phosphoprotein

Introduction

Numerous recent studies have suggested alterations in airway pH contribute to the pathophysiology of obstructive airway disease (reviewed in Ricciardolo *et al.*, 2004). Airway acidification caused by either exogenous acids (polluted air constituting an 'acid fog') (Honma *et al.*, 2000), endogenous acids (gastroesophageal reflux with microaspiration) (Ricciardolo, 2001; Hunt and Gaston, 2008), or as a consequence of airway inflammation (Kostikas *et al.*, 2002; Brunetti *et al.*, 2008) can reduce the pH of the airway and increase airway smooth muscle (ASM) contractility and airway resistance. Two major mechanisms mediating acid-induced bronchoconstriction have been identified. Termed the 'reflex' and 'reflux' mechanisms, both involve activation of afferent neurons by low pH to in turn promote neural release of bronchoconstricting agents such as ACh or kinins (Satoh *et al.*, 1993; Ricciardolo *et al.*, 1999).

Whether reduced pH in the airway microenvironment has direct effects on ASM is unknown. ASM contractile state is largely determined by activation of pro-contractile (e.g. the M_3 muscarinic acetylcholine receptor) and pro-relaxant (β_2-adrenoceptor) GPCRs expressed on ASM cells (Deshpande and Penn, 2006). Recently, a subfamily of GPCRs that are activated by a reduction in extracellular pH has been identified. The Gs-coupled receptors GPR4 and TDAG8 (T cell death-associated gene 8 or GPR65), and the Gq-coupled ovarian cancer G protein-coupled receptor 1 (OGR1 or GPR68) share similar sequence homology and were initially believed to be receptors for lysolipids (Seuwen *et al.*, 2006). However, the studies that ascribed lysolipid-binding properties to GPR4 and OGR1 have been retracted, and multiple studies have now asserted protons as the cognate ligand for these receptors. Interestingly, recent studies have characterized OGR1 activation in vascular smooth muscles (Tomura *et al.*, 2005b) and in ASM (Ichimonji *et al.*, 2010). The latter study identified OGR1 mRNA in human ASM, and demonstrated a reduction in extracellular pH to pH 6.3 stimulated calcium mobilization, p42/p44 phosphorylation and IL-6 production in cultured human ASM cells. Collectively, these studies prompted us to undertake a more comprehensive analysis of pH- and OGR1-dependent signalling in ASM and clarify: (i) whether ASM exhibits sensitivity to extracellular pH consistent with the profile of OGR1 responsiveness; and (ii) the capacity of OGR1 to mediate acid-induced contraction of ASM.

Methods

Materials

Antibodies against vasodilator-stimulated phosphoprotein (VASP) were from BD Biosciences (San Jose, CA, USA) and phospho-p42/p44 and phospho-PKB (Akt) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Fr122047 IRDye 680 or 800 secondary antibodies were from Rockland (Gilbertsville, PA, USA). myo-[2-3H(N)]-inositol (10–25Ci $mmol^{-1}$) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). All other materials were obtained from Sigma (St. Louis, MO, USA) or from previously identified sources (Deshpande *et al.*, 2008; Kong *et al.*, 2008).

Cell culture

Human ASM cultures were established from human primary bronchi or tracheae as described previously (Misior *et al.*, 2008). Data from cultures derived from tracheae and bronchi were similar, and were therefore analysed collectively. Third to eighth passage cells were plated at a density of 10^4 cells cm^{-2} in either 24-well [phosphoinositol (PI) hydrolysis and cAMP assays] 12-well (immunoblots), or 6-well (real-time PCR) plates and maintained in Ham's F12 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. The media was changed to serum-free media containing 5 μg mL^{-1} transferrin and 5 μg mL^{-1} insulin 24 h before stimulation, as described previously (Penn *et al.*, 1998).

Cellular cAMP accumulation

Human ASM cells grown to near confluence in 24-well plates were maintained in Ham's F12 media pH 8.0 in a 37°C, room air incubator for 3 h. To stimulate cells, media was replaced with Ham's F12 media pH 8.0 containing 1 mM IBMX and different amounts of dilute HCl to achieve media pH values of 8.0 to 6.4, for 10 min. Stimulation was terminated by aspiration of medium and quenching with 100% cold ethanol, after which cAMP was isolated and quantified by RIA as described previously (Penn *et al.*, 1998).

Assay of PI generation

Cells passaged into 24-well plates as described earlier were loaded with 2 μCi mL^{-1} *myo*-[^{3}H]-inositol for 18 h. Cells were then maintained in Ham's F12 media pH 8.0 in a 37°C, room air incubator for 1 h. Media were then exchanged with Ham's F12 media pH 8.0 containing 5 mM LiCl for 15 min and then stimulated for 30 min with different amounts of dilute HCl to reduce media pH. Reactions were terminated by replacing media with cold 20 mM formic acid. The inositol and PI fractions were separated by anion exchange chromatography, mixed with Ultima Gold and Ultima Flo AF scintillation fluid (Perkin Elmer, Waltham, MA, USA), respectively, and subjected to scintillation counting. To normalize for loading variability among wells, PI production was calculated as PI/(PI + inositol), and values reported as fold basal (vehicle-stimulated) production, as described previously (Deshpande *et al.*, 2007).

Immunoblotting

Cells were grown to near confluence in 12-well plates and growth-arrested as described earlier. Cells were then refed

Ham's F12 media pH 8.0 and placed in a 37°C, room air incubator for 3 h. In select experiments, cells were pretreated for 30 min with vehicle (control), 100 nM [des-Arg^{10}]-HOE 140 [B_1 bradykinin (BK) receptor antagonist], 100 nM HOE 140 (B_2 BK receptor antagonist), 100 nM L-733 060 (neurokinin NK_1 receptor antagonist), 100 nM GR 159897 (tachykinin NK_2 receptor antagonist), 10 μM indomethacin (non-selective COX inhibitor), 2 μM AG1478 (EGF receptor tyrphostin) or 2 μM AG1296 (PDGF receptor tyrphostin). Cells were stimulated with dilute HCl to achieve the indicated media pH, or with the indicated agents for 5 min or 0–15 min (time course experiments). Cells were then washed twice with ice-cold buffer (25 mM Tris, 150 mM NaCl, pH 8.0) then solubilized in a 25 mM Tris buffer (pH 8.0) containing 150 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM p-nitrophenyl phosphate, 1 mM benzamidine, 0.1 M PMSF and 1% (v/v) NP-40 (lysis buffer) for 30 min at 4°C. Following scraping, cell lysates were centrifuged at 13 200× *g* at 4°C for 10 min. Supernatants were collected then electrophoresed on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and subsequently probed with the indicated primary antibodies and secondary antibodies conjugated with infrared fluorophores.

Intracellular calcium [$(Ca^{2+})_i$] measurements

ASM cells grown on glass cover slips (Delta T dishes, Bioptechs, Butler, PA, USA) were washed and loaded with 5 μM Fura-2 AM in HBSS containing 10 mM HEPES, 11 mM glucose, 2.5 mM $CaCl_2$ and 1.2 mM $MgCl_2$ adjusted to pH 8.0 for 30 min at 37°C. The cells were then washed and maintained in the same HBSS pH 8.0 (lacking Fura-2). Calcium imaging was performed using Nikon fluorescent imaging system (Metafluor; Universal Imaging Corporation, Downingtown, PA, USA) as described previously (Deshpande *et al.*, 2003). The basal intracellular calcium was determined by superfusing the cells with HBSS pH 8.0 for 1–2 min. To assess the effect of reduced extracellular pH, the pH of HBSS was adjusted with HCl and cells were superfused with HBSS of varying pH (8.0–6.8) until steady-state calcium levels were achieved. The net calcium response was calculated by subtracting the basal from peak $[Ca^{2+}]_i$ upon agonist stimulation. The experiments were repeated using cells obtained from different donors and all the data are expressed as mean ± SEM values.

Real-time PCR analysis

Total RNA was isolated by standard procedures using Trizol (Invitrogen, Carlsbad, CA, USA), then 1 μg of RNA was converted to cDNA using TAQman reverse transcription reagents with Multiscribe reverse transcriptase (Applied Biosystems TaqMan® reverse transcription reagents Cat# N8080234). Human OGR1 (5'ACTTCGGCTACCTGCAGATCAA3', 5'AG CCCACGCTGATGTAGATGTT3'), GPR4 (5'ATACCACAGC TCACTGGCTTTC3', 5'TCATGGCTTTGGCTGTGCTGTT3'), TDAG8 (5'TGCCGTTGATCGGTATTTGGCT3', 5'TTGCATA GCCTGTACACGTCCT3'), G2A (5'TGCAACATCTACGTC AGCATCC3', 5'ATCTGCAGCATGTCAAAGCAGG3'), and *GAPDH* (5'CCCTTCATTGACCTCAACTACATGGT3', 5'TGAT GACAAGCTTCCCGTTCTCAG3') primers were used in a SYBR green PCR reaction (Quanta Biosciences PerfeCTa SYBR® Green FastMix ROX Cat#95073, Gaithersburg, MD, USA) using an ABI 7300 real-time PCR system. Briefly, a 50 μL reaction containing the TAQman RT reagents and 1 μg of RNA was incubated 25°C for 10 min, 48°C for 60 min, 95°C for 5 min, then 4°C overnight in a Bio-Rad C1000 thermal cycler. 2 μL of the cDNA reaction was then added to a PCR plate with the primer set and amplified: 50°C × 2 min, 95°C × 10 min, followed by 40 PCR cycles at 95°C (15 s) and 60°C (1 min). Results were normalized to *GAPDH* using the comparative C_t method. The threshold cycle C_t is defined as the cycle number at which the ΔRn crosses a software-generated threshold defined as 10 SDs above baseline (during cycles 3–15). The C_t is linearly proportional to the logarithm of the input copy number. Negative controls included GAPDH amplification using RT reactions in which reverse transcriptase was omitted.

siRNA-mediated knockdown of OGR1 in ASM cells

For OGR1 (ON-TARGETplus SMARTpool # L-005591-00) (Thermo Scientific, Lafayette, CO, USA), 2 μg of siRNA duplexes or scrambled (control) sequence (5'-GCG CGC UUU GUA GGA UUC GdTdT-3') were mixed in 1X siRNA buffer, and 60 mm dishes of human ASM cultures (plated 24 h previously at a density of 10^4 cells cm^{-2}) were transfected using Dharmafect transfection reagent (Thermo Scientific) as per the manufacturer's instructions. Twenty-four hours later, cells were passaged onto 12-well (immunoblot analyses of pH-dependent signalling) or 6-well (real-time PCR analysis of OGR1 mRNA levels) plates, or onto cover slips (Ca^{2+} mobilization) or collagen-coated Stripwell microplate 1 × 8 wells (contraction analysis) (Corning Inc., Corning, NY, USA) for assays performed 72 h later, corresponding to the period of peak OGR1 knockdown.

Magnetic twisting cytometry (MTC)

Dynamic changes in cell stiffness were measured as an indicator of contraction of isolated ASM cells using the MTC technique as described previously (An *et al.*, 2006; Deshpande *et al.*, 2010). Briefly, Arg-Gly-Asp (RGD) coated ferrimagnetic microbeads bound to adherent cells were magnetized horizontally and then twisted in a vertically aligned homogeneous magnetic field that was varying sinusoidally in time. This sinusoidal twisting magnetic field caused both a rotation and a pivoting displacement of the bead: such forced bead motions are, in turn, impeded by internal stresses developed by the cell (Fabry *et al.*, 2001). Lateral bead displacements in response to the resulting oscillatory torque were detected with a spatial resolution of ~5 nanometer (nm), and the ratio of specific torque to bead displacements was computed and expressed as the cell stiffness in units of Pascal (Pa) per nm. For each individual ASM cell, baseline stiffness was measured for the first 60 s, and changes in cell stiffness in response to either histamine (10 μM) or reductions in media pH were measured continuously for the next 240 s.

Statistical analysis

Data are presented as mean ± SEM values, from *n* experiments in which each experiment was performed using a different culture derived from a unique donor. Individual data points from a single experiment were calculated as the

mean value from three replicate observations for PI accumulation experiments. For immunoblot analyses, band intensities representing signals from secondary antibodies conjugated with infrared fluorophores were visualized and directly quantified using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA) as described previously (Billington *et al.*, 2005). Phospho-VASP signals were expressed as a percentage of total VASP (i.e. [(50 KDa band intensity)/(46 Kda + 50 KDa band intensities) × 100], and p-p42/p44 as fold-basal values. Because in some experiments, basal (vehicle-stimulated) p-PKB values were either very low or nonexistent, data are presented as fold maximal value. Statistically significant differences among groups were assessed by either ANOVA with Fisher's protected least significant difference *post hoc* analysis or by *t*-test for paired samples, with *P*-values < 0.05 sufficient to reject the null hypothesis using GraphPad Prism software (La Jolla, CA, USA).

Nomenclature for all drugs and molecular targets conforms to *British Journal of Pharmacology's* Guide to Receptors and Channels (Alexander *et al.*, 2011).

Results

Preliminary studies using real-time PCR analysis of ASM mRNA abundance revealed C_t values for OGR1 ranging from ~21 to 25 (mean ± SEM OGR1 C_t 23.39 ± 1.12; GAPDH 15.55 ± 0.36, n = 11). Corresponding C_t values for GPR4, TDAG8 and G2A were typically >4 log units higher (GPR4 27.29 ± 2.69; TDAG8 28.84 ± 1.04; G2A 29.57 ± 0.99; n = 6–10). Consistent with these data, we were able to observe a product after reverse transcriptase- (RT) PCR for OGR1 in human ASM, but little or no product for TDAG8 or GPR4 (Figure 1). Interestingly, PCR products for each of the receptors were readily observed in human airway epithelium grown in an air/liquid interface.

In our recent characterization of the putative proton-sensing GPCR *GPR4* in renal medullary collecting duct cells (Sun *et al.*, 2010), we observed activity of the receptor at pH 7.4, as well as progressive stimulation of both cAMP and phosphorylation of the cAMP-dependent protein kinase, PKA, substrate VASP when media pH was reduced from pH 8.0 to pH 6.8. Similarly, in the original characterization of OGR1 (Ludwig *et al.*, 2003), OGR1 stably expressed in CCL39 cells possessed constitutive activity at pH 7.4 and

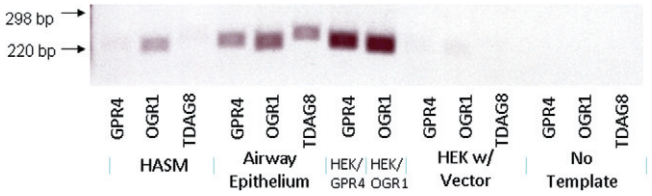

Figure 1

RT-PCR analysis of proton-sensing receptor expression in human airway cells. RT-PCR was performed as described in Methods using mRNA from human ASM, human airway epithelium, or HEK293 cells expressing recombinant GPR4 or OGR1.

exhibited responsiveness starting at pH 8.0 and peaking at ~pH 7.0–6.8. To assess the sensitivity of human ASM cells to modest reductions in extracellular pH within this same range, ASM cultures were first maintained for 3 h in culture media pH 8.0. Subsequent addition of small volumes of dilute HCl to culture media resulted in a small (approximately two- to threefold) increase in intracellular cAMP (Figure 2A) and a clear (but somewhat variable among different ASM cultures) induction of phosphorylation of the PKA substrate VASP (Figure 2B). However, a more profound phosphorylation of p42/p44, as well as PKB phosphorylation was observed in a 'dose'- and time-dependent manner (Figure 2B–F). Similarly, levels of p-VASP and p-p42/p44 (but p-PKB only minimally) were increased by treatment with 100 nM BK, a pro-contractile, Gq-coupled receptor expressed in ASM.

Whereas the BK-stimulated induction of p-VASP was reversed by COX inhibition, acid-induced p-VASP was variably affected by COX inhibition. Although COX inhibition attenuated p-VASP induction in some ASM cultures (not shown), the effect was absent or modest in most cultures examined, relative to that observed on the BK-induced increase in p-VASP (Figure 3A).

Because of the novelty of the concept of pH-/proton-activated GPCRs, we considered numerous design and technical issues that could influence the results. In preliminary experiments, we found that pH-dependent changes in second messenger and signalling events are robust phenomena. Acute increases in phosphorylation of p42/p44, PKB and VASP induced by reductions in pHo occurred similarly within a given line when experiments utilized different types of (pH-adjusted) media, including DMEM, Ham's F12 or HBSS buffer. Moreover, addition artefacts, or local or order effects did not account for the responses. To minimize addition artefacts, media pH was altered by addition of HCl diluted first in media or buffer, such that its addition to the well did not create high local concentrations of protons (i.e. local currents of very low pH).

The potential for acute reductions in extracellular pH to promote proteolytic cleavage of membrane or matrix products, which in turn activate signalling pathways, was also considered. Combined pretreatment of cells with B_1 and B_2 BK receptor antagonists inhibited signalling by BK but not by reduced pHo; similarly, neurokinin receptor antagonists also had no effect, and pretreatment with protease inhibitors had no effect (not shown). Because certain GPCRs have been shown to activate pro-mitogenic or survival signalling via transactivation of receptor tyrosine kinases, we examined whether acid-induced p42/p44 and PKB activation in human ASM involved such a mechanism. We have previously used pretreatment with the tyrphostins AG1478 and AG1296 to inhibit EGF and PDGF tyrosine kinase activity, respectively, and examine GPCR-mediated receptor kinase transactivation in ASM (Krymskaya *et al.*, 2000). As shown in Figure 3B and C, pretreatment with either AG1478 or AG1296 had no effect on acid-induced p42/p44 or PKB phosphorylation.

Further signalling analyses (Figure 4) also demonstrate a 'dose-dependent' increase in phosphoinositide hydrolysis and Ca^{2+} mobilization in ASM cells as media/buffer pH was progressively reduced, with maximal effects observed at ~pH 7.0–6.8.

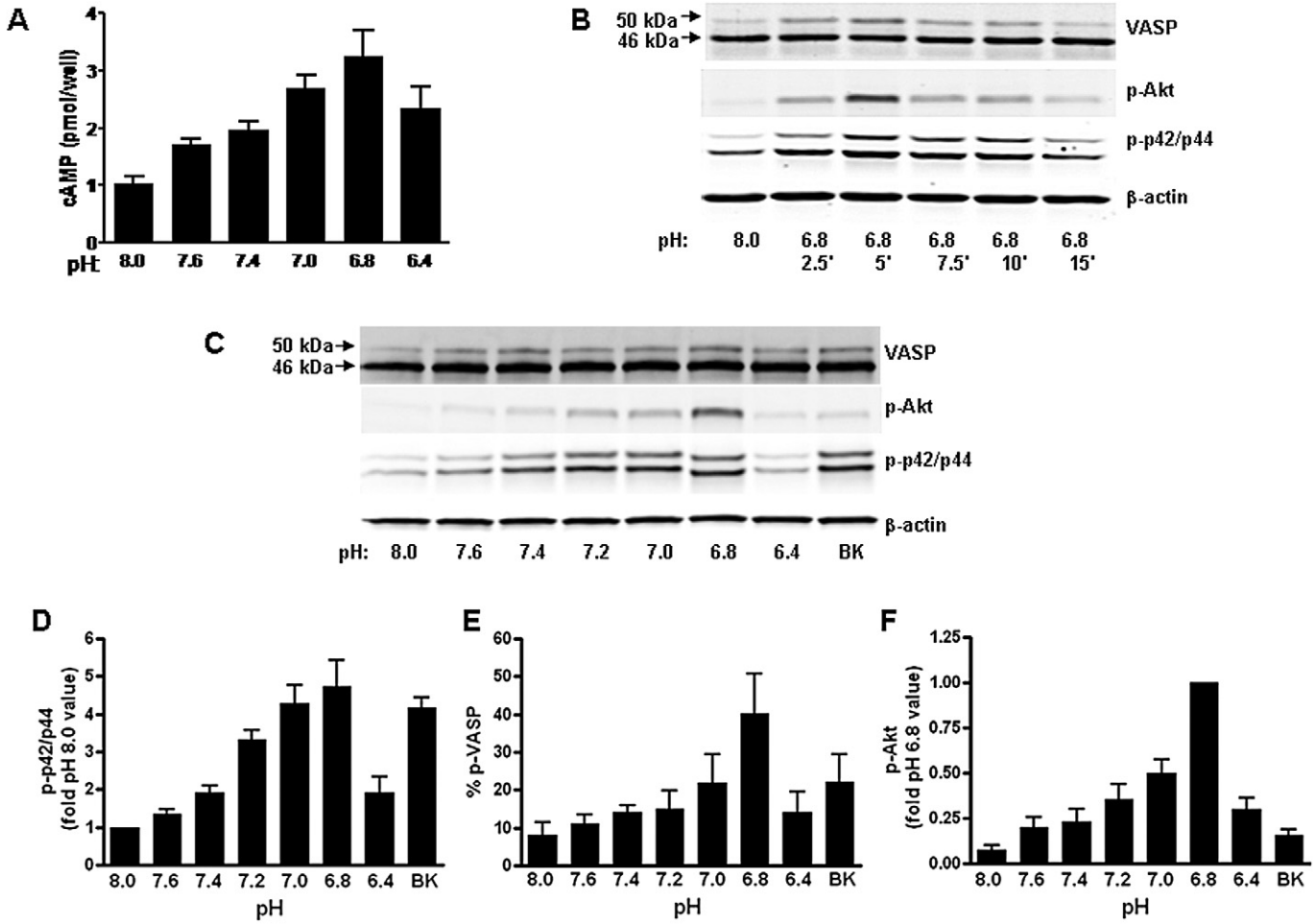

Figure 2

Time- and dose-dependent effect of acid stimulation of ASM on VASP, PKB (Akt), and p42/p44 phosphorylation. Human cultured ASM cells maintained in Ham's F12 media pH 8.0 were stimulated as indicated with 100 nM BK or HCl to achieve the indicated media pH. cAMP was subsequently isolated as quantified by RIA (A) or lysates harvested and immunoblots (B,C) detecting expression of total VASP, phospho-p42/p44, or phospho- ($pSer^{473}$) PKB were generated as described previously (Kong *et al.*, 2006). The 50 KDa species of VASP is specifically induced by PKA phosphorylation in human ASM (Guo *et al.*, 2005). (A) Depicts mean + SEM values (n = 5) for cAMP accumulation after 10 min stimulation by indicated media pH; (B) depicts the time course of stimulation with media pH 6.8, and (C) 5 min stimulation with variable media pH or BK. Mean ± SEM values from six to seven independent experiments are presented in (D, E).

Because our collective findings suggested that the signalling induced by reducing the pHo could be mediated by OGR1, we examined the effect of OGR1 knockdown on acid-induced signalling. As in our analysis of GPR4 (Sun *et al.*, 2010), we first validated the ability of siRNA to knockdown OGR1 protein by demonstrating in HEK293 cells knockdown of stably expressed HA-tagged OGR1 with OGR1 siRNA oligos (Figure 5A). A similar knockdown of endogenous OGR1 in ASM cells was suggested by real-time PCR analysis of OGR1 siRNA (no useful antibodies exist for endogenous OGR1); knockdown based on differences in C_t values normalized to GAPDH values was calculated as 81 ± 9% (mean ± SEM, n = 8). This knockdown was associated with a significant reduction in acid-induced p-p42/p44 (Figure 5B, C). Acid-induced VASP phosphorylation was also significantly inhibited (Figure 5B, D). Although variability in the increased level of p-VASP induced was again observed among cultures, siRNA-mediated OGR1 knockdown resulted in a 39 ± 13% decrease in VASP phosphorylation. Conversely, OGR1 knockdown had no effect on PKB phosphorylation (Figure 5B, E). In addition, Ca^{2+} mobilization induced by reducing pHo was significantly inhibited by OGR1 knockdown (Figure 5F, G).

Lastly, to demonstrate the physiological relevance of our findings, the effect of reductions in pHo and the role of OGR1 in the ASM cell contractile state were assessed using the MTC technique. With MTC, magnetic beads are bound to cells, and stimulus-invoked changes in the ratio of specific torque to lateral bead displacements are computed and expressed as the cell stiffness (in Pa nm^{-1}), providing a readout for contractile function of ASM cells (An *et al.*, 2002). As shown in Figure 6A and B, small, progressive reductions in media pH caused appreciable increases in ASM stiffness. To demonstrate the role of OGR1 in this contractile effect, siRNA-mediated knockdown of OGR1 was performed as mentioned earlier. As

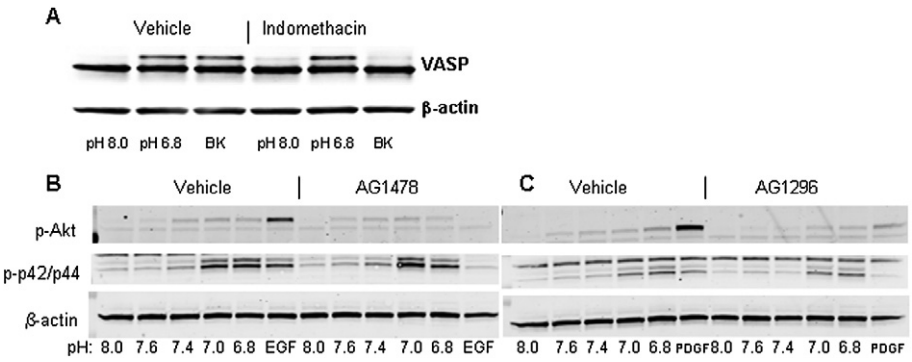

Figure 3

Effect of COX inhibition, receptor tyrosine kinase tyrphostins on acid-induced signalling in ASM. Cultured human ASM cells were maintained in Ham's F12 media pH 8.0, then pretreated for 30 min with either vehicle or 10 μM indomethacin (A), or vehicle and either AG1478 (left) or AG1296 (right) (2 μM each) (B). Cells were then stimulated as indicated for 5 min with HCl to achieve the indicated media pH, or with 100 nM BK, 10 ng mL^{-1} EGF, or 10 ng mL^{-1} PDGF-BB. Lysates were subsequently harvested and the protein/phoshoprotein levels assessed by immunoblotting as described previously (Guo *et al.*, 2005). For each experiment, similar results were obtained in at least two additional experiments.

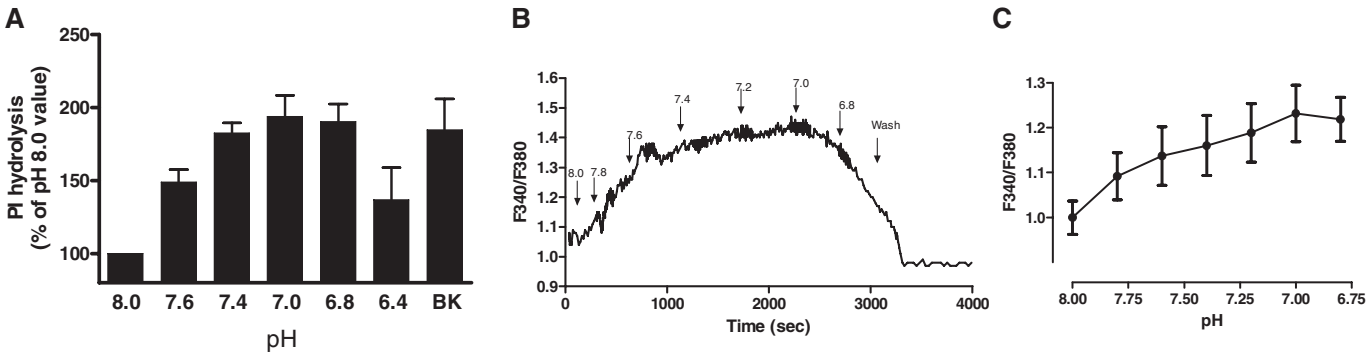

Figure 4

Reduced pHo stimulates PI hydrolysis and Ca^{2+} mobilization in human ASM cells. (A) PI hydrolysis after 30 min stimulation with media of pH 8.0 to 6.4 or 100 nM BK; $n = 3$, mean ± SEM values. (B) Single-cell calcium mobilization with mean + SEM values ($n = 6$ cells in each field) plotted in (C).

shown in Figure 7A–C, OGR1 knockdown did not affect baseline or histamine-induced increases in ASM stiffness, but significantly inhibited that induced by reductions in pHo.

Discussion

In the present study, we demonstrated the ability of modest reductions in extracellular pH to promote multiple signalling events, as well as contraction, in human ASM cells. A role for the putative proton-sensing OGR1 in acid-induced activation of p42/p44, PKA, as well as Ca^{2+} mobilization and contraction, was demonstrated by the ability of siRNA-mediated OGR1 knockdown to significantly attenuate each of these responses.

Numerous studies have proposed a role for airway pH in regulating airway resistance, yet have pointed to neural mechanisms as critical to the airway response to airway acidification. Animal models employing citric acid inhalation demonstrate activation of pulmonary sensory neurons as an important mechanism mediating the bronchoconstricting effect of reduced airway pH (Satoh *et al.*, 1993; Ricciardolo *et al.*, 1999). Proton activation of vanilloid receptor subtype 1 (TRPV1) on sensory neurons induces depolarization of afferents that in turn stimulate the local release of kinins that induce ASM contraction (Ricciardolo *et al.*, 1999; Gu and Lee, 2006). In addition, acid-sensing ion channels (ASICs) are also expressed on pulmonary sensory neurons, and modest reductions in extracellular pH have been shown to depolarize these nerves (Gu and Lee, 2006). However, a recent study suggests that ASICs may play a role in pH-induced ASM relaxation via a mechanism independent of sensory nerves, perhaps involving ASIC activation on airway epithelium or smooth muscle (Faisy *et al.*, 2007). Our findings presented herein suggest that OGR1 expressed on ASM cells represents a direct target whereby airway acidification mediates ASM contraction.

Under normal physiological conditions, the pH of the airway fluid lining appears to be slightly alkaline (Ricciardolo *et al.*, 2004). For healthy subjects, early studies reported mean pH values from exhaled breath condensate (EBC) (Hunt *et al.*,

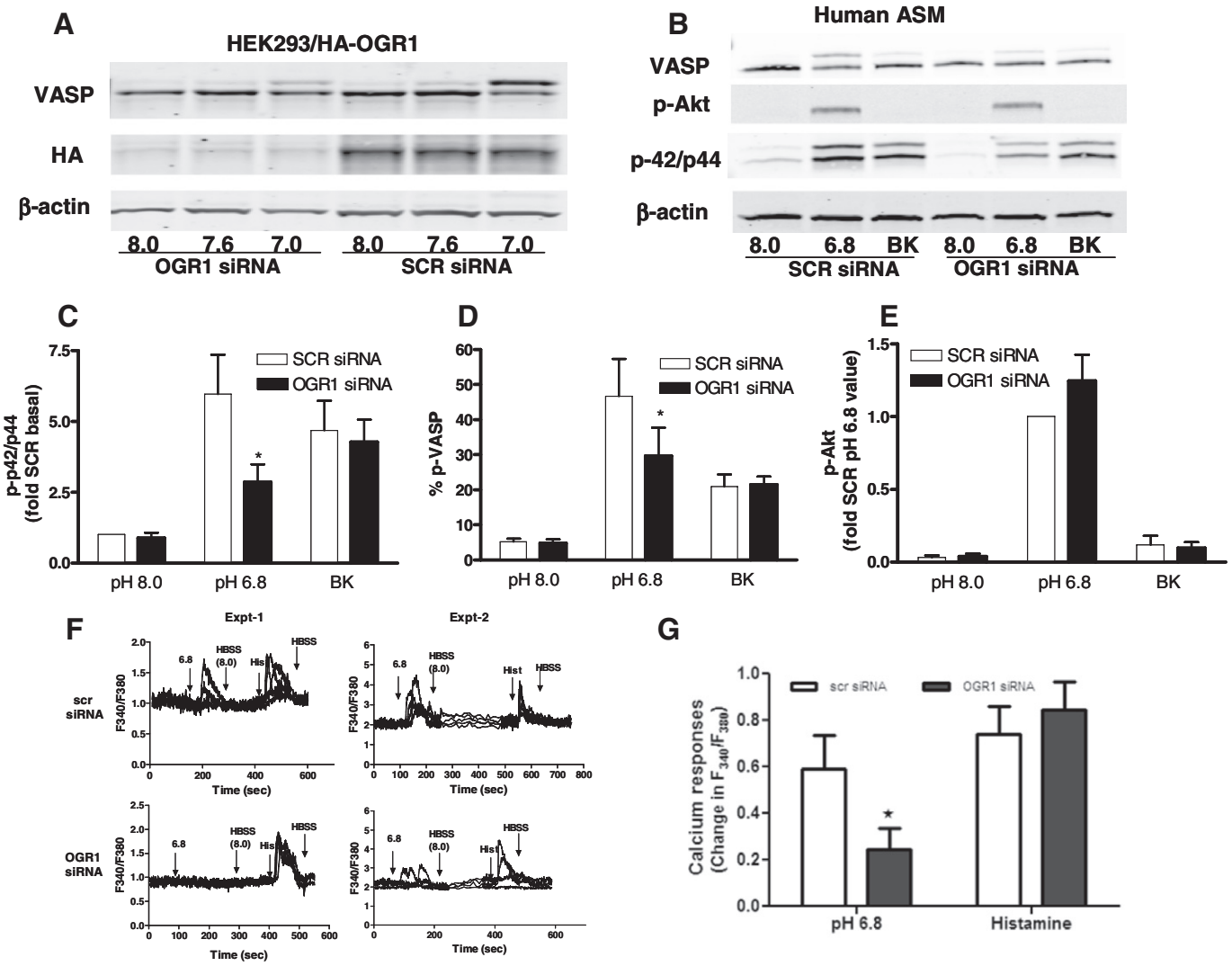

Figure 5

Effects of OGR1 knockdown on acid-induced signalling in ASM cells. (A) siRNA targeting OGR1 knocks down the expression of HA-tagged OGR1 expressed in 293 cells. (B) Transfection of OGR1 siRNA in human ASM attenuated acid-induced p42/p44, PKA activation. Five-minute stimulation as indicated: BK = 100 nM bradykinin. (C–E) Mean ± SEM values from six independent experiments that assessed the effect of OGR1 knockdown on the increase in p-VASP, p-p42/p44, and p-PKB (Akt) induced by reducing pHo (F) Representative experiments demonstrating effect of OGR1 knockdown on Ca^{2+} mobilization induced by reduction in pHo, with mean ± SEM values from six experiments presented in (G) *$P < 0.05$, SCR siRNA vs. OGR1 siRNA group.

2000; Vaughan *et al.*, 2003) and tracheobronchial mucous (Metheny *et al.*, 1999) of 7.7 and 7.8, respectively. A recent study conducted by the Severe Asthma Research Programme (Liu *et al.*, 2011) reported that nonasthmatic control subjects have mean EBC pH values of 7.9 [interquartile range (IQR) 7.40–8.20] while stable severe asthmatics maintained long term on high dose inhaled or systemic corticosteroids have similar values (8.02; IQR 7.61–8.41).

Numerous studies provide evidence that the airway is subject to variations in pH. Importantly, several of the events that promote reductions in airway pH are associated with ASM contraction and obstructive lung diseases. 'Acid fog' is a prominent environmental factor (the pH in floating fog over Kushiro City, Japan averages < 5.0) that can cause acute bronchoconstriction and is associated with an increased incidence of asthma (Folinsbee, 1989). There is a long history of research exploring the relationship between gastroesophageal reflux disease (and microaspiration of gastric contents) and asthma (reviewed in Ricciardolo, 2001). Perhaps most importantly, inflammation is known to produce reductions in local pH, and patients with chronic obstructive pulmonary disease or bronchiectasis (Kostikas *et al.*, 2002), or asthmatics undergoing acute exacerbations (Koschel, 1980; Hunt *et al.*, 2000; Kostikas *et al.*, 2002) or poorly controlled (Koschel, 1980; Kostikas *et al.*, 2002; Kodric *et al.*, 2007) have significantly lower airway pH values than those of healthy controls. Moreover, Kosttokas *et al.* (Kostikas *et al.*, 2002) reported that pH values assessed for asthmatics were significantly correlated

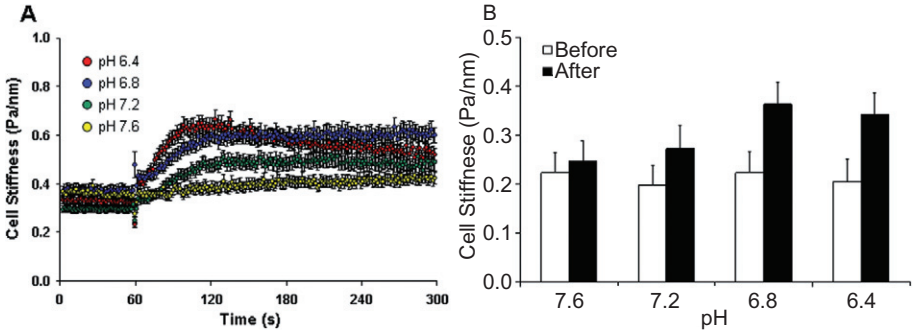

Figure 6

Effect of reduced pHo on the contractile state of human ASM cells measured by MTC. A modest and progressive reduction in pHo increased the stiffness of adherent human ASM cells. (A) Represents time-dependent response, and (B) represents the steady-state cell stiffness before and after stimulation with the indicated pHo. Data are presented as geometric mean + SEM values from three independent experiments; at least 400 cells were assessed for each stimulating condition.

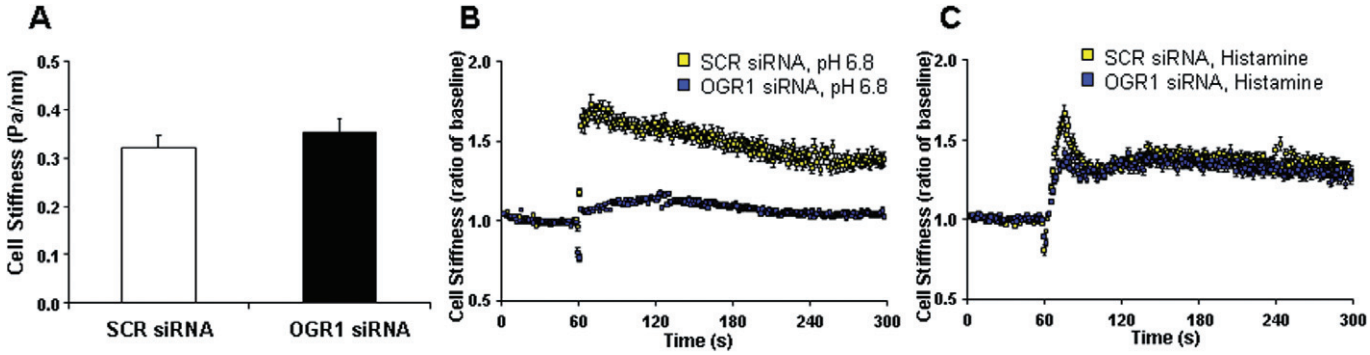

Figure 7

Role of OGR1 in ASM contractility induced by reduced pHo. Cells transfected with siRNA targeting OGR1 as in Figure 5 demonstrate no differences in baseline cell stiffness (A), but significantly reduced stiffness development once they were stimulated by a reduction in pHo to 6.8 (B), relative to values recorded for cells transfected with scrambled (SCR) control siRNA. (C) Response to 10 μM histamine in siRNA-transfected cells. Data in (A) are presented as geometric mean ± SEM; data in (B) and (C) are normalized to respective baseline stiffness and presented as mean ± SEM. Data are from four independent experiments (n = 365–469 cells).

with either sputum eosinophilia or neutrophilia, and with oxidative stress. A more recent study (Kostikas *et al.*, 2011) reported that asthmatics who were well-controlled had mean EBC pH values of 7.44 (IQR 7.34–7.57) whereas asthmatics who were poorly controlled had values of 7.14 (IQR 7.05–7.21). In addition, Hunt *et al.* (Hunt *et al.*, 2000) measured EBC pH values 2 log orders lower in asthmatic patients admitted for an acute exacerbation compared with values measured in patients admitted for acute, non-respiratory diseases (pH 5.23 ± 0.21 vs. pH 7.65 ± 0.20). Interestingly, treatment of these asthmatics with inhaled corticosteroids resulted in a normalization of airway pH. Collectively, results from these studies strongly suggest that: (i) airway pH values in healthy subjects or stable asthmatics estimated using either EBC or induced sputum tend to be slightly alkaline but demonstrate a range of pH values in the airway that overlap with the activation profile of OGR1; and (ii) airway pH is inversely related to asthma control and airway inflammation. Moreover, these findings imply that modest reductions in airway pH associated with the induction of airway inflammation provide a stimulus for OGR1-mediated bronchoconstriction.

Results from the current study demonstrate that ASM is exquisitely sensitive to reductions in extracellular pH, exhibiting progressive activation of p42/p44, PKA, PKB, PI hydrolysis and Ca^{2+} mobilization with modest step reductions in media pH. With the exception of PKB, each of these signals is at least partially dependent on OGR1, as suggested by the effect of OGR1 knockdown. Moreover, the induction of p-p42/p44, PI hydrolysis, and Ca^{2+} mobilization appears to follow the 'dose-dependent' activation profile of OGR1 previously demonstrated for recombinantly expressed OGR1, whereby receptor activation increased progressively as media pH was decreased from 8.0 to ~7.0/6.8, with signalling waning with further pH reduction. Why signalling tends to wane at pH values < 6.8 is unclear; possibilities include differences in OGR1 conformation induced at pH < 6.8, or pH effects independent of OGR1 activation.

Most importantly, in the present study, ASM cell tension development was also shown to be highly sensitive to small reductions in pHo, thus demonstrating the physiological relevance of extracellular pH on ASM contractile state, and presumably the relevance of modest variations in airway pH on bronchomotor tone and airway resistance. That OGR1 mediates acid-induced ASM contraction is suggested by the inhibitory effect of OGR1 knockdown on ASM cell tension development, consistent with the observed effect of knockdown on acid-induced Ca^{2+} mobilization.

Also intriguing is the ability of OGR1 to promote phosphorylation of the PKA substrate VASP in response to reductions in pHo. Although a similar effect is evident for the B_2 BK receptor via a COX-dependent mechanism, we found that COX inhibition had a variable and often minimal effect on the VASP phosphorylation induced by reductions in pHo. These findings raise the possibility that OGR1-depdendent PKA activation can be independent of Gq activation, possibility reflecting direct coupling to Gs. Of note, other GPCRs such as the IP receptor (Lawler *et al.*, 2001), Edg-3 (Windh *et al.*, 1999) and vasopressin V_{1B} receptor (Orcel *et al.*, 2009) exhibit the ability to couple to both Gs and Gq proteins.

Although our findings suggest that OGR1 is a pro-contractile ASM GPCR capable of responding to an airway microenvironment that exists in those with inflammatory airway disease, a considerable amount of work will be required to ascertain the true role of OGR1 and ASM OGR1 in obstructive lung disease pathology. Studies employing more integrative models will need to dissect the relative contribution of acid-invoked neural mechanisms vs. ASM OGR1 activation in regulating bronchomotor tone, and consider the prevalence of other proton-sensing GPCRs, including OGR1, on other airway cell types, both resident and infiltrating. Multiple proton-sensing GPCRs appear to be expressed on airway epithelia (current study) as well as on inflammatory cells (Mogi *et al.*, 2009; Tomura *et al.*, 2005a), raising the possibility of an extremely complex role of proton-sensing GPCRs in both airway inflammation and airway resistance.

Acknowledgements

This study was funded by the National Institute of Health Heart, Lung, and Blood Institute HL108071 (RBP) and [HL087560] (DAD). HS is supported by the Interdisciplinary Training Program in Muscle Biology training grant AR007592. SSA is also supported by the American Asthma Foundation (Sandler:108183).

Conflict of interest

The authors declare no conflicts of interest.

References

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

An SS, Laudadio RE, Lai J, Rogers RA, Fredberg JJ (2002). Stiffness changes in cultured airway smooth muscle cells. Am J Physiol Cell Physiol 283: C792–C801.

An SS, Fabry B, Trepat X, Wang N, Fredberg JJ (2006). Do biophysical properties of the airway smooth muscle in culture predict airway hyperresponsiveness? Am J Respir Cell Mol Biol 35: 55–64.

Billington CK, Kong KC, Bhattacharyya R, Wedegaertner PB, Panettieri RA, Chan TO *et al.* (2005). Cooperative regulation of p70S6 kinase by receptor tyrosine kinases and G protein-coupled receptors augments airway smooth muscle growth. Biochemistry 44: 14595–14605.

Brunetti L, Francavilla R, Tesse R, Fiermonte P, Fiore FP, Lore M *et al.* (2008). Exhaled breath condensate cytokines and pH in pediatric asthma and atopic dermatitis. Allergy Asthma Proc 29: 461–467.

Deshpande DA, Penn RB (2006). Targeting G protein-coupled receptor signaling in asthma. Cell Signal 18: 2105–2120.

Deshpande DA, Walseth TF, Panettieri RA, Kannan MS (2003). CD38/cyclic ADP-ribose-mediated Ca2+ signaling contributes to airway smooth muscle hyper-responsiveness. FASEB J 17: 452–454.

Deshpande DA, Pascual RM, Wang SW, Eckman DM, Riemer EC, Funk CD *et al.* (2007). PKC-dependent regulation of the receptor locus dominates functional consequences of cysteinyl leukotriene type 1 receptor activation. FASEB J 21: 2335–2342.

Deshpande DA, Theriot BS, Penn RB, Walker JK (2008). Beta-arrestins specifically constrain beta2-adrenergic receptor signaling and function in airway smooth muscle. FASEB J 22: 2134–2141.

Deshpande DA, Wang WC, McIlmoyle EL, Robinett KS, Schillinger RM, An SS *et al.* (2010). Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. Nat Med 16: 1299–1304.

Fabry B, Maksym GN, Butler JP, Glogauer M, Navajas D, Fredberg JJ (2001). Scaling the microrheology of living cells. Phys Rev Lett 87: 148102.

Faisy C, Planquette B, Naline E, Risse PA, Frossard N, Fagon JY *et al.* (2007). Acid-induced modulation of airway basal tone and contractility: role of acid-sensing ion channels (ASICs) and TRPV1 receptor. Life Sci 81: 1094–1102.

Folinsbee LJ (1989). Human health effects of exposure to airborne acid. Environ Health Perspect 79: 195–199.

Gu Q, Lee LY (2006). Characterization of acid signaling in rat vagal pulmonary sensory neurons. Am J Physiol Lung Cell Mol Physiol 291: L58–L65.

Guo M, Pascual RM, Wang S, Fontana MF, Valancius CA, Panettieri RA Jr *et al.* (2005). Cytokines regulate beta-2-adrenergic receptor responsiveness in airway smooth muscle via multiple PKA- and EP2 receptor-dependent mechanisms. Biochemistry 44: 13771–13782.

Honma S, Tanaka H, Teramoto S, Igarashi T, Abe S (2000). Effects of naturally-occurring acid fog on inflammatory mediators in airway and pulmonary functions in asthmatic patients. Respir Med 94: 935–942.

Hunt JF, Gaston B (2008). Airway acidification and gastroesophageal reflux. Curr Allergy Asthma Rep 8: 79–84.

Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TA *et al.* (2000). Endogenous airway acidification. Implications for asthma pathophysiology. Am J Respir Crit Care Med 161: 694–699.

Ichimonji I, Tomura H, Mogi C, Sato K, Aoki H, Hisada T *et al.* (2010). Extracellular acidification stimulates IL-6 production and Ca(2+) mobilization through proton-sensing OGR1 receptors in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 299: L567–L577.

Kodric M, Shah AN, Fabbri LM, Confalonieri M (2007). An investigation of airway acidification in asthma using induced sputum: a study of feasibility and correlation. Am J Respir Crit Care Med 175: 905–910.

Kong KC, Billington CK, Gandhi U, Panettieri RA Jr, Penn RB (2006). Cooperative mitogenic signaling by G protein-coupled receptors and growth factors is dependent on G(q/11). FASEB J 20: 1558–1560.

Kong KC, Gandhi U, Martin TJ, Anz CB, Yan H, Misior AM *et al.* (2008). Endogenous Gs-coupled receptors in smooth muscle exhibit differential susceptibility to GRK2/3-mediated desensitization. Biochemistry 47: 9279–9288.

Koschel K (1980). A hormone-independent rise of adenosine 3′, 5′ -monophosphate desensitizes coupling of β-adrenergic receptors to adenylate cyclase in rat glioma C6-cells. Eur J Biochem 108: 163–169.

Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S (2002). pH in expired breath condensate of patients with inflammatory airway diseases. Am J Respir Crit Care Med 165: 1364–1370.

Kostikas K, Papaioannou AI, Tanou K, Giouleka P, Koutsokera A, Minas M *et al.* (2011). Exhaled NO and exhaled breath condensate pH in the evaluation of asthma control. Respir Med 105: 526–532.

Krymskaya VP, Orsini MJ, Eszterhas A, Benovic JL, Panettieri RA, Penn RB (2000). Potentiation of human airway smooth muscle proliferation by receptor tyrosine kinase and G protein-coupled receptor activation. Am J Respir Cell Mol Biol 23: 546–554.

Lawler OA, Miggin SM, Kinsella BT (2001). Protein kinase A-mediated phosphorylation of serine 357 of the mouse prostacyclin receptor regulates its coupling to G(s)-, to G(i)-, and to G(q)-coupled effector signaling. J Biol Chem 276: 33596–33607.

Liu L, Teague WG, Erzurum S, Fitzpatrick A, Mantri S, Dweik RA *et al.* (2011). Determinants of exhaled breath condensate pH in a large population with asthma. Chest 139: 328–336.

Ludwig MG, Vanek M, Guerini D, Gasser JA, Jones CE, Junker U *et al.* (2003). Proton-sensing G-protein-coupled receptors. Nature 425: 93–98.

Metheny NA, Stewart BJ, Smith L, Yan H, Diebold M, Clouse RE (1999). pH and concentration of bilirubin in feeding tube aspirates as predictors of tube placement. Nurs Res 48: 189–197.

Misior AM, Yan H, Pascual RM, Deshpande DA, Panettieri, RA Jr, Penn RB (2008). Mitogenic effects of cytokines on smooth muscle are critically dependent on protein kinase A and are unmasked by steroids and cyclooxygenase inhibitors. Mol Pharmacol 73: 566–574.

Mogi C, Tobo M, Tomura H, Murata N, He XD, Sato T *et al.* (2009). Involvement of proton-sensing TDAG8 in extracellular acidification-induced inhibition of proinflammatory cytokine production in peritoneal macrophages. J Immunol 182: 3243–3251.

Orcel H, Albizu L, Perkovska S, Durroux T, Mendre C, Ansanay H *et al.* (2009). Differential coupling of the vasopressin V1b receptor through compartmentalization within the plasma membrane. Mol Pharmacol 75: 637–647.

Penn RB, Panettieri RA Jr, Benovic JL (1998). Mechanisms of acute desensitization of the β_2AR-adenylyl cyclase pathway in human airway smooth muscle. Am J Resp Cell Mol Biol 19: 338–348.

Ricciardolo FL (2001). Mechanisms of citric acid-induced bronchoconstriction. Am J Med 111 (Suppl 8A): 18S–24S.

Ricciardolo FL, Rado V, Fabbri LM, Sterk PJ, Di Maria GU, Geppetti P (1999). Bronchoconstriction induced by citric acid inhalation in guinea pigs: role of tachykinins, bradykinin, and nitric oxide. Am J Respir Crit Care Med 159: 557–562.

Ricciardolo FL, Gaston B, Hunt J (2004). Acid stress in the pathology of asthma. J Allergy Clin Immunol 113: 610–619.

Satoh H, Lou YP, Lundberg JM (1993). Inhibitory effects of capsazepine and SR 48968 on citric acid-induced bronchoconstriction in guinea-pigs. Eur J Pharmacol 236: 367–372.

Seuwen K, Ludwig MG, Wolf RM (2006). Receptors for protons or lipid messengers or both? J Recept Signal Transduct Res 26: 599–610.

Sun X, Yang LV, Tiegs BC, Arend LJ, McGraw DW, Penn RB *et al.* (2010). Deletion of the pH sensor GPR4 decreases renal acid excretion. J Am Soc Nephrol 21: 1745–1755.

Tomura H, Mogi C, Sato K, Okajima F (2005a). Proton-sensing and lysolipid-sensitive G-protein-coupled receptors: a novel type of multi-functional receptors. Cell Signal 17: 1466–1476.

Tomura H, Wang JQ, Komachi M, Damirin A, Mogi C, Tobo M *et al.* (2005b). Prostaglandin I(2) production and cAMP accumulation in response to acidic extracellular pH through OGR1 in human aortic smooth muscle cells. J Biol Chem 280: 34458–34464.

Vaughan J, Ngamtrakulpanit L, Pajewski TN, Turner R, Nguyen TA, Smith A *et al.* (2003). Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. Eur Respir J 22: 889–894.

Windh RT, Lee MJ, Hla T, An S, Barr AJ, Manning DR (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. J Biol Chem 274: 27351–27358.