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Ovarian Cancer G protein-coupled receptor-1 signaling bias dictates anti-contractile effect of benzodiazepines on airway smooth muscle

Dominic R. Villalba[1], Arun K. Jannu[1], Elham Javed[1], Isha Dandekar[2], Ruping Wang[3], Deepak A. Deshpande[1], Steven S. An[2], Reynold A. Panettieri Jr.[2], Dale D. Tang[3], Raymond B. Penn[1] and Ajay P. Nayak[1*]

Abstract

Background We recently reported that the ovarian cancer G protein-coupled receptor-1 (OGR1) can be pharmacologically biased with specific benzodiazepines to couple with distinct heterotrimeric G proteins in human airway smooth muscle (ASM) cells. Lorazepam stimulated both G_s and G_q signaling via OGR1, whereas sulazepam only stimulated G_s signaling in ASM cells. The present study sought to determine the effects of sulazepam and lorazepam on contraction of human precision cut lung slices (hPCLS), and detail the biochemical mechanisms mediating these effects.

Methods Models of histamine (His) -stimulated contraction included imaging of ex vivo human precision cut lung slices (hPCLS) and Magnetic Twisting Cytometry (MTC) analysis of human ASM cell stiffness. To explore mechanisms of regulation, we examined effects on myosin light chain (pMLC) phosphorylation and PKA activity in primary human ASM cultures, as well as actin cytoskeleton integrity as defined by changes in the ratio of F to G actin assessed by immunofluorescence.

Results In a dose-dependent manner, sulazepam relaxed His-contracted hPCLS and reduced baseline cell stiffness. Lorazepam did not relax His-contracted hPCLS, and only at a maximal dose (100 µM) did lorazepam relax baseline cell stiffness. The G_s-biased ligand sulazepam stimulated PKA activity as evidenced by significant induction of VASP and HSP20 phosphorylation, which was associated with significant inhibition of His-induced pMLC phosphorylation. Conversely, the balanced ligand lorazepam did not significantly increase HSP20 phosphorylation or VASP phosphorylation and did not significantly inhibit His-induced MLC phosphorylation. Sulazepam was also able to inhibit histamine induced F-actin formation.

Conclusions The G_s-biased OGR1 ligand sulazepam relaxed contracted ASM in both tissue- and cell- based models, via inhibition of MLC phosphorylation in a PKA-dependent manner and through inhibition of actin stress fiber formation. The relative inability of the balanced ligand lorazepam to influence ASM contractile state was likely due to competitive actions of concomitant G_q and G_s signaling.

Keywords Benzodiazepines, Airway smooth muscle, OGR1, GPCR

*Correspondence:
Ajay P. Nayak
ajay.nayak@jefferson.edu
Full list of author information is available at the end of the article

Introduction

Conditions that result in low airway pH, such as changes in environment (acid fog) [1], microaspiration of acid reflux [2, 3] or inflammation [4, 5], contribute to the neural mechanisms associated with airway contraction [6]. Low pH can activate afferent neurons to stimulate release of bronchoconstrictors such as acetylcholine (ACh) [7, 8]. Exhaled breath condensate (EBC) pH values show a significant negative correlation with inflammatory markers associated with chronic obstructive pulmonary disease (COPD) and asthma, with normalization of pH following resolution of airway inflammation by treatment with inhaled corticosteroids [3, 4]. Initial studies in our lab centered around the hypothesis that low pH in the airway plays a pathogenic role in obstructive airway disease by directly activating the proton-sensing G protein-coupled receptors (GPCRs) on airway smooth muscle (ASM). Studies revealed that the ovarian cancer G protein-coupled receptor 1 (OGR1; *aka* GPR68) is the predominant proton-sensing GPCR in human ASM cells, and incremental reductions in extracellular pH (pH_o) stimulate OGR1 in ASM cells to increase cell stiffness [9].

In subsequent studies, we determined specific benzodiazepines, namely lorazepam and sulazepam, to be agonists of OGR1 [10], a discovery that helped overcome the inherent difficulties of studying a receptor whose cognate ligand is the (promiscuous) proton [6]. We also determined that "biased" signaling of OGR1 can occur, thus forming the basis to selectively "tune" OGR1. More specifically, the benzodiazepine lorazepam behaved in a balanced manner, stimulating mobilization of intracellular calcium and activation of p42/p44 in a G_q dependent manner, but also eliciting generation of cAMP suggesting recruitment of G_s signaling via OGR1 in ASM. Conversely, sulazepam stimulated only G_s signaling in ASM, defining sulazepam as G_s-biased [11]. Moreover, sulazepam, unlike lorazepam and reduced pH_o, demonstrated resistance to agonist-induced OGR1 desensitization [12]. More recently, we demonstrated that prophylactic treatment of mice with sulazepam inhibits development of airway hyperresponsiveness in a murine model of allergen-induced asthma [13]. Collectively, these studies underscore the potential therapeutic value of benzodiazepines in regulating ASM contractile state.

However, the mechanisms mediating the anti-contractile effects of benzodiazepines on ASM contractile state remain poorly defined. In the present study, we characterize the effects of lorazepam and sulazepam on ASM contractile state in both human precision cut lung slices (hPCLS) and cultured human ASM cells, and detail the mechanisms underlying these effects.

Materials and Methods

Antibodies and other reagents

Antibodies against the regulatory phosphorylated myosin light chain 2 (pMLC; threonine 18, serine 19) and vasodilator-stimulated phosphoprotein (VASP) were purchased through Cell Signaling Technology (Danvers, MA). Phosphorylated heat shock protein 20 (pHSP20; serine 16) was purchased through Abcam (Boston, MA). β-actin antibody, lorazepam, and isoproterenol (ISO) were purchased from Millipore Sigma (St. Louis, MO). Sulazepam was procured from Specs (Zoertermeer, Netherlands).

Human ASM (hASM) cell culture

Primary human ASM cells (passages 2–6) were established from human tracheae obtained from de-identified non-asthmatic donors as described previously [12, 14, 15]. Cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) and maintained in a 37 °C incubator with 5% CO_2 until confluence and subsequently starved in serum-free medium as per [12, 14].

Human precision cut lung slices (hPCLS)

Human precision cut lung slices were generated from donor-derived human lung cores as described previously [16] and used as a model for studying regulation of bronchoconstriction by sulazepam and lorazepam. The use of deidentified human lung cores, and human ASM cell cultures, for experimental purposes was approved by the Rutgers University and Thomas Jefferson University Institutional Review Boards. Briefly, hPCLS were generated by cutting lung cores transversely at 350 µm and slices were maintained in Ham's F12 media supplemented with Primocin® (Invivogen, San Diego, CA) at 37 °C in an incubator supplemented with 5% CO_2. Airways were washed and utilized in experiments detailed below. Multiple airways, in PCLS derived from 2 distinct donors, were examined.

To examine the effectiveness of benzodiazepines in regulating relaxation, hPCLS were stimulated with His (1 µM) for 15 min and subsequently treated with increasing doses of either lorazepam or sulazepam (0.1 nM – 100 µM), or 1 µM (ISO), with 10 min of incubation for each dose. Images were taken prior to and after His treatment, then after incubation of each dose of sulazepam or lorazepam. Data analysis was performed as described previously [16].

Magnetic twisting cytometry (MTC)

Changes in ASM cell stiffness, a surrogate measure of ASM contractile state, were measured using MTC as described previously [11]. Briefly, RGD-coated ferrimagnetic microbeads were functionalized to the cytoskeleton

(CSK) of adherent hASM cells through cell surface integrin receptors, magnetized horizontally to the cell plating, and twisted in a vertically aligned magnetic field that varied sinusoidally in time. The aforementioned magnetic field causes a rotation and pivoting movement of the microbeads which is inhibited by the cell stiffening or stressing. Spatial resolution from oscillatory torque of lateral bead displacement was ~5 nm, and the ratio of said torque to bead displacement gave us our cell stiffness in Pascal units/nm and was expressed as a ratio of induced stiffness to baseline stiffness or as percent relaxation from precontracted cell stiffness. For each individual ASM cell effects of lorazepam and sulazepam were assessed by first measuring the baseline for 60 s then treating with either sulazepam or lorazepam (25, 50, or 100 μM) for the following 300 s.

Immunoblotting

To examine MLC regulation, human ASM cultures were grown to near confluence and subject to serum deprivation for 72 h. Cells were then pretreated with sulazepam, lorazepam, vehicle (0.1% DMSO), or ISO for 10 min and subsequently treated with His for an additional 10 min. For these studies, His was used as contractile agonist given the variability of response to MCh that occurs with the progressive loss of m3 muscarinic acetylcholine receptors that occurs after passage 2 of human ASM cultures [15, 17, 18]. Cells were lysed in 1× RIPA solution, and cell lysates subjected to western blotting procedure as described previously [19]. Following electrophoresis and transfer onto nitrocellulose membranes, membranes were incubated for 1 h at RT with Tris-buffered saline containing 0.2% Tween-20 (TBST) containing 3% bovine serum albumin (BSA; w/v). Membranes were then incubated at 4 °C overnight with antibodies against pMLC (1:1000 in 3% BSA), VASP (1:5000 in 3% BSA), pHSP20 (1:1000 in 3% BSA), and β-actin (1:50,000 in 3% BSA). The following day, membranes were washed briefly with TBST and incubated at RT for 1 h with secondary antibodies (1:10,000 dilution in TBST) conjugated to infrared dyes (Li-Cor; Lincoln, NE). Immunoreactive bands were quantified using the Li-Cor Image Studio™.

Regulation of the actin cytoskeleton

Primary hASM cells were plated on coated coverslips overnight, starved for 72 h and then pretreated with sulazepam for 10 min. Cells were then treated with basal media or His for 5 min. Cells were fixed in 4% PFA solution and the stained utilizing Phalloidin Rhodamine (F-Actin; 1:100 dilution) and Alexa Fluor 488 DNase I (G-actin; 1:50 dilution). Fluorescence images of F and G actin stains were visualized on a Leica DMI 6000 microscope. Cell fields free of cell debris were randomly selected, and the fluorescent intensity was analyzed using the NIH image J software. F/G actin ratios were calculated as follows: the intensity of Phalloidin over the intensity of DNase I. The investigator choosing the fields was blinded to control or treated groups.

Data presentation and statistical analysis

For hPCLS experiments data are represented as means ± standard error of the mean (SEM) for *n* observations where *n* reflects different airways. For MTC experimental data represents as mean ± SEM for *n* observations for each time point. In these data *n* reflects different cells. Data from experiments that utilized hASM cell cultures in immunoblotting are represented as means ± SE for *n* observations where *n* observations reflect experiments employing *n* different human donor-derived cultures. Because vehicle- treated cells often exhibit an undetectable level of phosphorylated HSP20, values for band intensity of pHSP20 from immunoblot analyses were normalized to values determined for the (positive control) ISO-stimulated condition. PKA-mediated phosphorylation of VASP at serine 157 (ser157) causes a mobility shift on electrophoresis gels with pVASP appearing at 50 kDa and unphosphorylated VASP appearing at 46 kDa [12, 16], therefore VASP was represented as a % VASP shift (pVASP band intensity/(pVASP band intensity + unphosphorylated VASP intensity)*100). Statistical analysis was performed with GraphPad Prism (San Diego, CA) software. Statistically significant differences between experimental and control groups were assessed with One-way ANOVA with Bonferroni's post-hoc analysis of multiple comparisons, with $p < 0.05$ being sufficient to establish significance between groups.

Results

Benzodiazepine ligands of OGR1 modulate ASM contraction in human precision cut lung slices (hPCLS)

Our studies initially focused on assessing the ability of benzodiazepines to relax ASM tissue ex vivo. We utilized the hPCLS system to examine the efficacy of lorazepam and sulazepam in reversing His-induced contraction of airway lumen in isolated slices (Fig. 1). Sulazepam exhibited a significant dose-dependent reversal of His-induced airway closure (E_{max} = 84 + 14% relaxation and -logIC_{50} = 6.4e-005), comparable to albeit less potent than that of ISO (Fig. 1C). Conversely, lorazepam was largely ineffective, producing but a modest inhibitory effect at the highest concentration (100 μM) tested, that was not statistically significant (Fig. 1B).

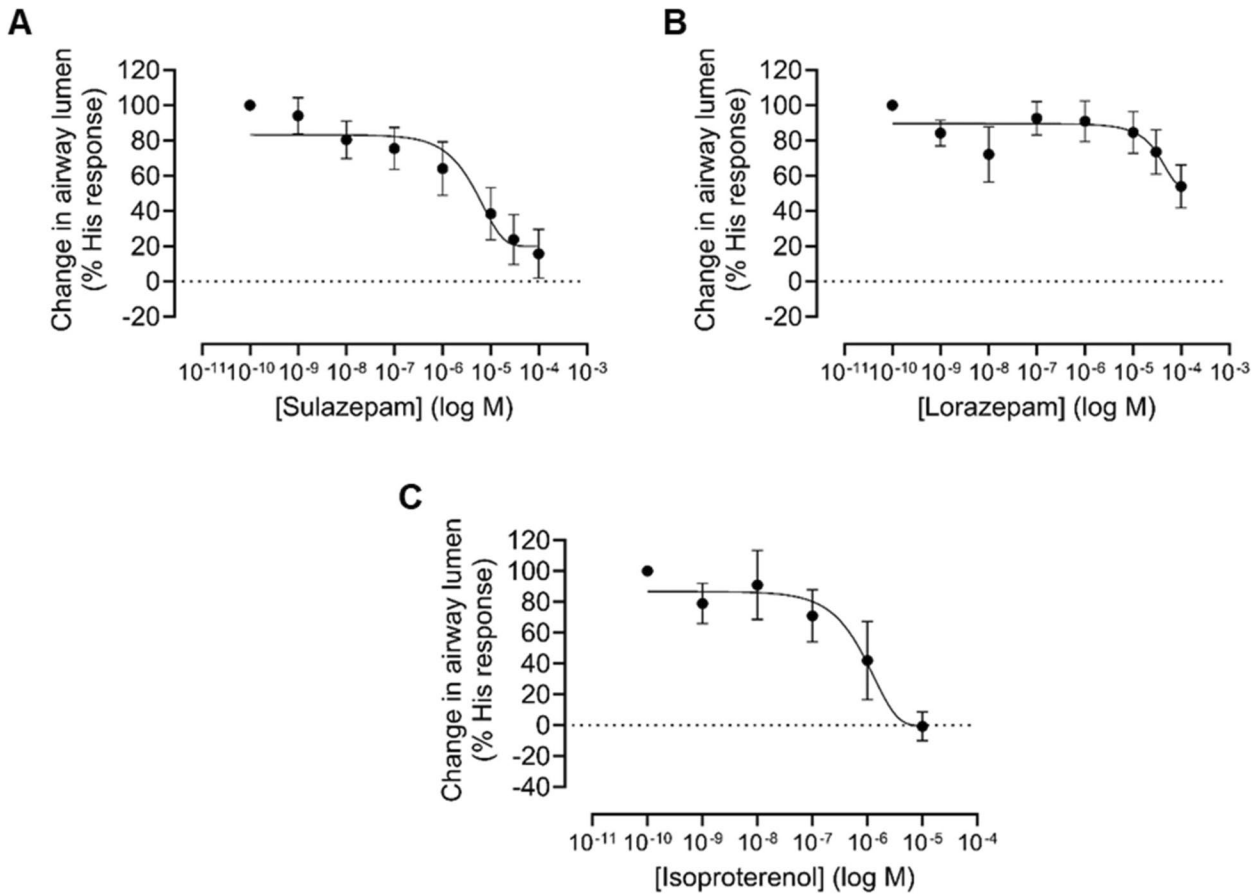

Fig. 1 Airway relaxation profile of benzodiazepines in human precision cut lung slices (hPCLS). Pre-contracted human airways isolated from lung cores from distinct donors subjected to sequential dosing with **A**) Sulazepam (1 nM to 100 µM), **B**) Lorazepam, or **C**) isoproterenol control (1 nM to 10 µM). E_{max} = 84 ± 14% relaxation and -logIC_{50} = 6.3e-005 for sulazepam treated slices, E_{max} = 46 ± 12% relaxation and -logIC_{50} = 2.4e-005 for lorazepam treated slices, and E_{max} = 100.7 ± 9.3% relaxation and -logIC_{50} = 1.5 e-005 for isoproterenol treated slices. Data represent % change (± SEM values) in airway lumen area relative to normalized to 100% response (His, 10 µM). Emax is presented as % relaxation which was calculated as 100 – (% histamine response). Data generated from *n* = 10 airways for sulazepam, *n* = 16 airways for lorazepam, *n* = 15 airways for isoproterenol. All airways were generated from

Benzodiazepine ligands of OGR1 modulate ASM cell stiffness

Because PCLS represents an integrated system with possible influences of other cell types such as epithelial cells or mast cells embedded within PCLS sections, we examined the efficacy of benzodiazepines in regulating ASM function using MTC which employs isolated primary human ASM cells. Consistent with our previous study [11], sulazepam reversed baseline ASM cell stiffness in a dose-dependent manner (Fig. 2). Lorazepam at concentrations of 25 and 50 µM did not alter ASM cell stiffness. A modest decrease in cell stiffness was observed with the 100 µM dose.

Mechanism of ASM relaxation by benzodiazepines

Regulation of MLC

We next assessed the ability of sulazepam and lorazepam to regulate MLC phosphorylation state, a key determinant in smooth muscle contractile state (Fig. 3A and B). MLC phosphorylation is subject to inhibition by PKA, and G_s-coupled GPCR activation of PKA activity, such as that mediated by beta-2-adrenoceptors (β_2ARs), can relax contracted ASM [20]. Treatment of hASM cells with His significantly increased MLC phosphorylation compared to the vehicle condition. Pretreatment with lorazepam had little effect on His-induced MLC phosphorylation, although lorazepam alone (at 50 and 100

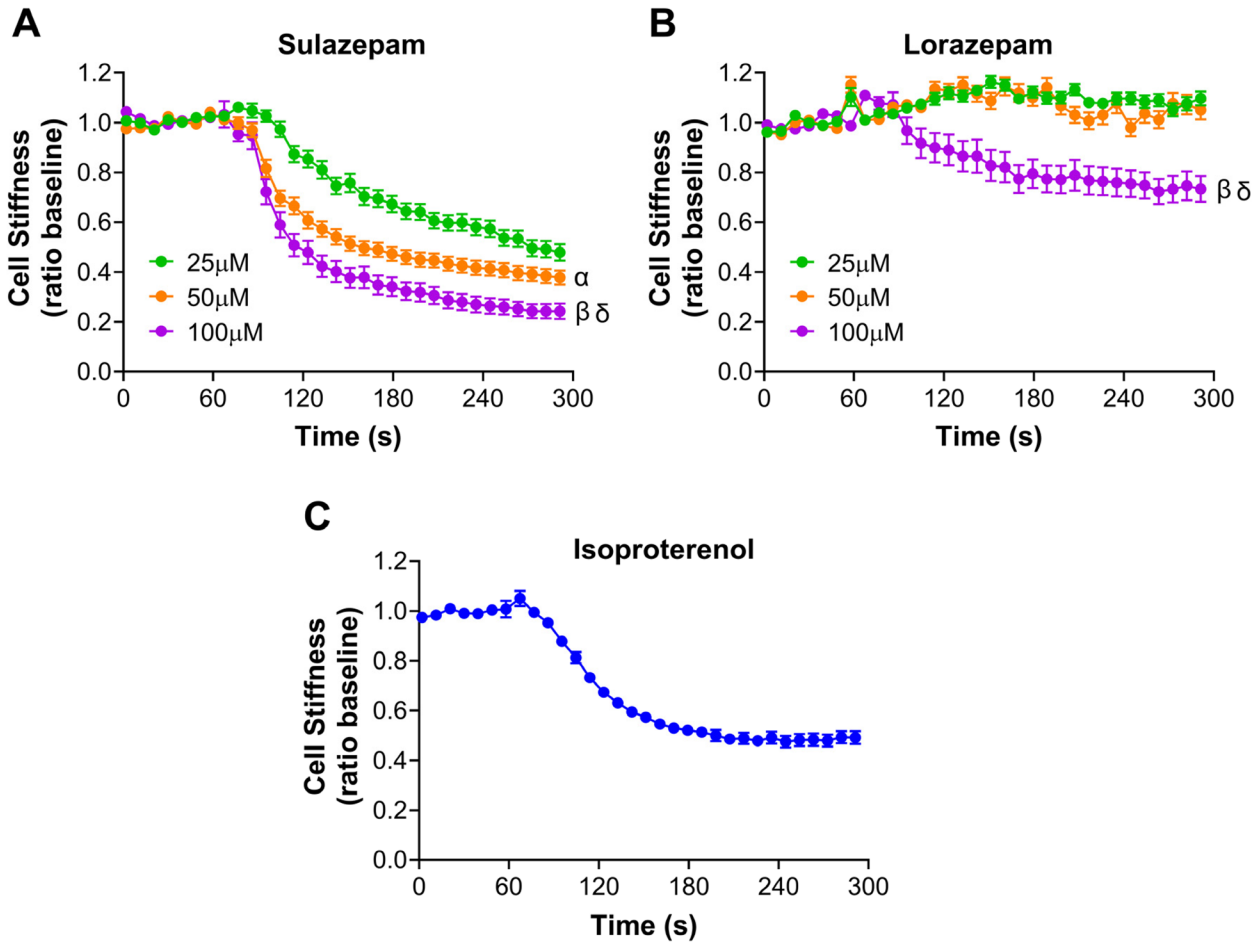

Fig. 2 Benzodiazepine-induced change in ASM cell stiffness by magnetic twisting cytometry (MTC). Primary human ASM cells treated with (25, 50, or 100 μM) sulazepam (**A**), lorazepam (**B**), or isoproterenol (1 μM) (**C**) and changes in baseline cell stiffness examined by MTC. Data represent mean ± SE values from $n = 50–292$ replicates. Statistical significance ($P < 0.05$) for comparisons between 25 and 50 μM (α), 25 and 100 μM (β) and 50 and 100 μM (δ) measured at 300 secs relative to baseline cell stiffness

μM) caused MLC phosphorylation, consistent with its ability to stimulate G_q, calcium mobilization, and PKC [9, 11]. Pretreatment with sulazepam or ISO significantly reduced His-induced MLC phosphorylation (65 ± 8.1% of His-induced pMLC vs. 27 ± 5.2% of His-induced pMLC respectively).

We next assessed the ability of lorazepam or sulazepam to regulate PKA activity as evidenced by (PKA substrate) HSP20 (Fig. 4A and C) and VASP phosphorylation (Fig. 4B and D). Treatment of hASM cells with sulazepam led to a significant increase in HSP20 phosphorylation after treatment with either vehicle or His (38 ± 6.7% of Iso-induced phosphorylation of HSP20 and 54 ± 9.5% of ISO-induced phosphorylation of HSP20). However, lorazepam had a modest, variable effect and failed to significantly induce HSP20 phosphorylation. Moreover, sulazepam was able to significantly increase VASP phosphorylation (29 ± 5.6% VASP shift), whereas lorazepam was not (Fig. 4B and D). As expected, pretreatment with ISO led to a strong induction of pHSP20 and VASP shift (64 ± 4.0%).

Sulazepam regulation of actin cytoskeletal dynamics

Previous studies demonstrate that changes in F-actin to G-actin ratio (F/G-actin ratio) can contribute to both contraction and ASM relaxation. To explore a potential additional mechanism of sulazepam-mediated ASM relaxation, we assessed whether pretreatment of ASM cells with sulazepam was capable of regulating F/G-actin dynamics (Fig. 5). We observed an increase in F/G-actin ratio in ASM cells stimulated with His alone (1.8 ± 0.17-fold vehicle). Cells pretreated with sulazepam resulted in a significant reduction in the His-induced F/G-actin ratio (reduced to 1.3 ± 0.25-fold vehicle).

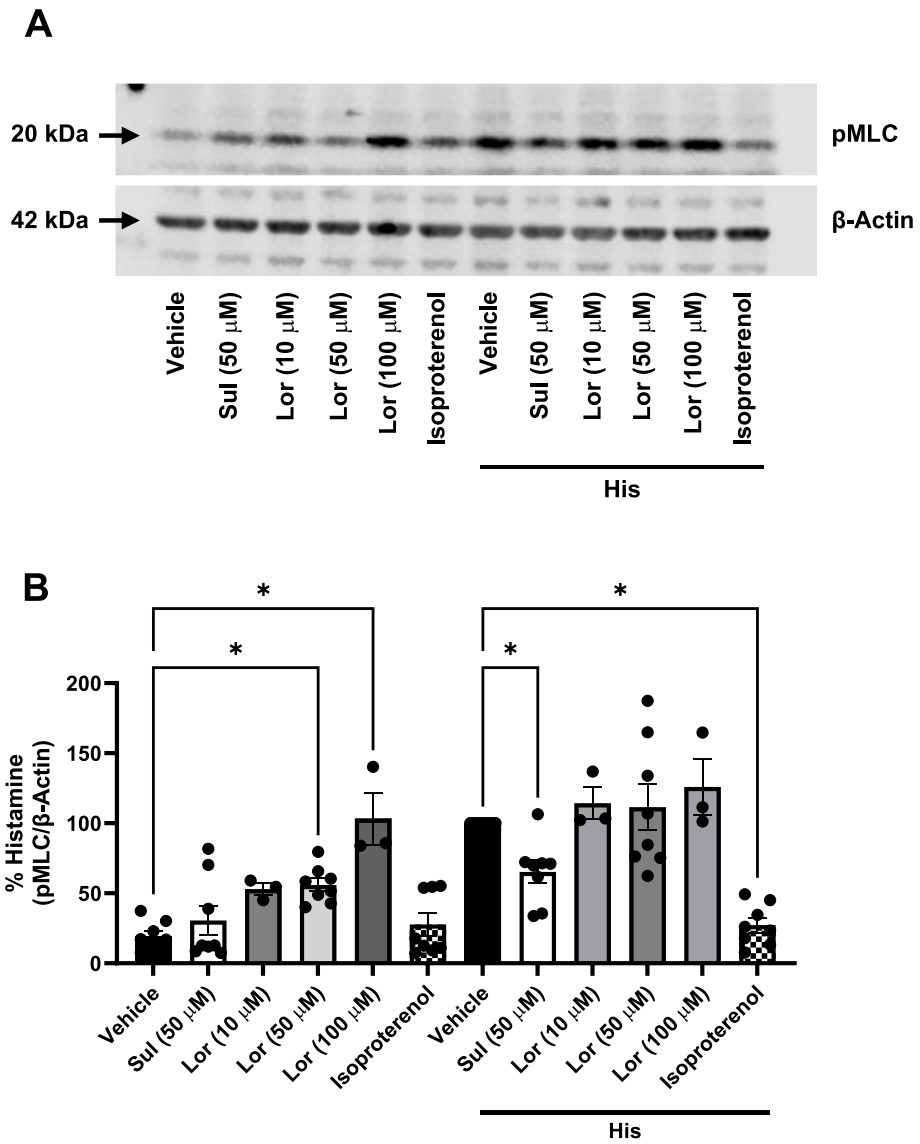

Fig. 3 Regulation of pharmacomechanical coupling by benzodiazepines. **A** Representative immunoblot of pMLC and β actin of lysates from primary hASM cells pretreated with sulazepam (50 μM; Sul), lorazepam (10–100 μM; Lor), or isoproterenol (1 μM) for 10 min and subsequently stimulated with His (1 μM) for 10 min (Vehicle- 0.1% DMSO). **B** Bar graph of immunoblot analysis of pMLC integrated intensity signal normalized to β actin loading control. Data represent mean ± SE values from $n = 3–8$ independent hASM cultures (*ns* = not significant; * $P < 0.05$)

Discussion

OGR1 belongs to the proton-sensing receptor family of GPCRs [6]. Initial studies implicated the 'proton' as the cognate ligand for this receptor [21]; Huang et al. subsequently determined specific benzodiazepines capable of activating OGR1 [10, 11]. We subsequently established disparate signaling capabilities among these specific benzodiazepines. Lorazepam was able to stimulate OGR1 coupling to G_q proteins, as highlighted by studies utilizing the G_q inhibitor YM254890 to significantly inhibit lorazepam induced mobilization of calcium and p42/p44 activation, as well as drive coupling to G_s, where it modestly caused generation of cAMP and downstream PKA activation. Sulazepam stimulated activation of only G_s-mediated signals. By utilizing the MTC technique we characterized the ability of these OGR1-targeting benzodiazepines to regulate ASM contractile state, with sulazepam but not lorazepam exhibiting a relaxant effect. To rule out relaxant effects of other targets of these benzodiazepines, our previous work also looked at $GABA_A$ channels in the ASM mediated responses to lorazepam. Experiments utilizing flumazenil and bicuculline could

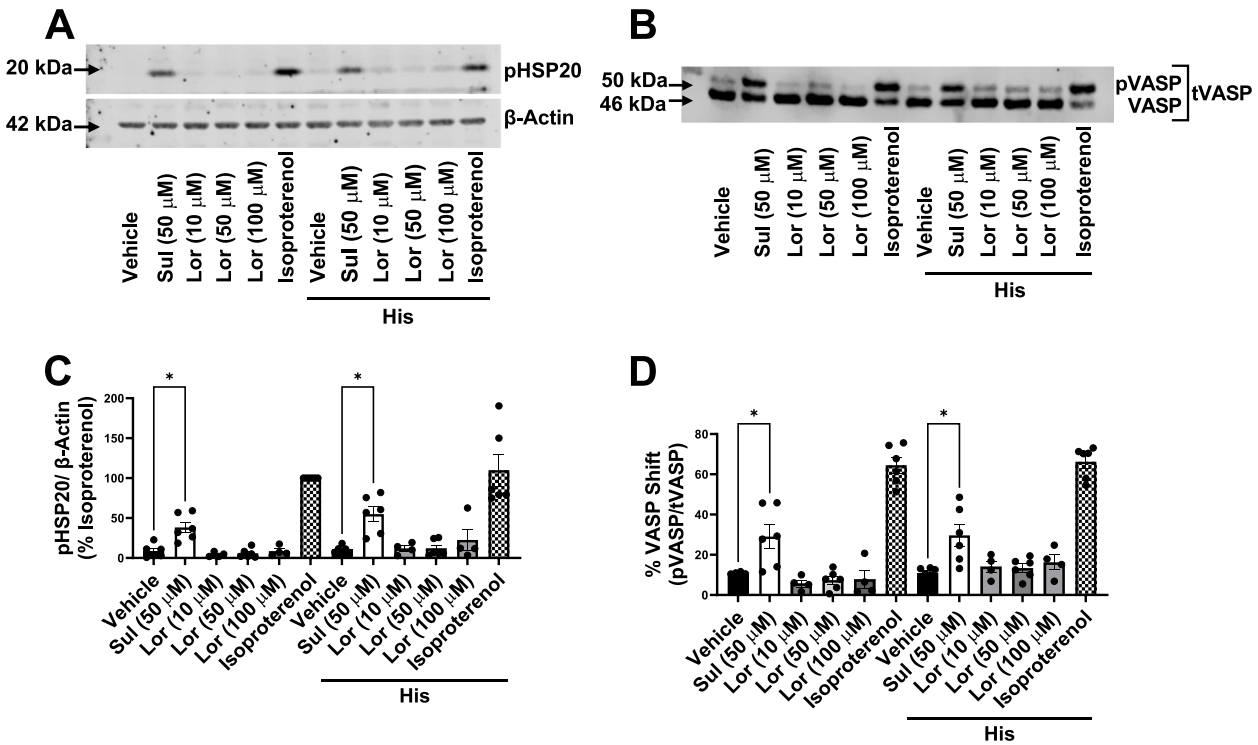

Fig. 4 PKA activation associated with benzodiazepine-induced regulation of pMLC. **A** Representative immunoblots of pHSP20 and β actin in hASM pretreated with either sulazepam (50 μM; Sul), lorazepam (10—100 μM; Lor), or isoproterenol (1 μM; Iso) for 10 min and subsequently stimulated with histamine (1 μM; His) for 10 min (Veh = vehicle- 0.1% DMSO). **B** Representative immunoblots of total VASP in hASM pretreated with either Sul (50 μM), Lor (10—100 μM), or Iso (1 μM) for 10 min and subsequently stimulated with His (1 μM) for 10 min. **C** Bar graph of immunoblot analysis of pHSP20 signal normalized to β actin loading controls. Data represent mean ± SE values from $n = 4$–6 independent hASM cultures (*ns* = not significant; * $P < 0.05$). **D** Bar graph of immunoblot analysis of VASP Shift (50 kDa pVASP/pVASP + 46 kDa VASP expressed as a percentage). Data represent mean ± SEM values from $n = 4$–6 independent hASM cultures

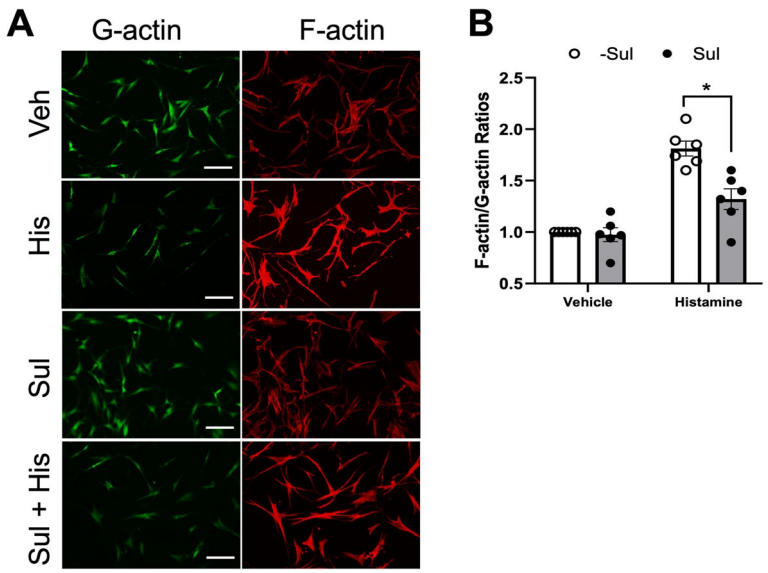

Fig. 5 Effect of sulazepam on His-induced actin polymerization in human ASM cells. **A** Representative images of ASM cells pretreated with sulazepam (Sul; 50 μM) for 10 min and stimulated with histamine (His; 10 μM) for 5 min and imaged for F/G-actin ratios. (Veh = vehicle- 0.05% DMSO) **B** Bar graph of analysis of F/G actin ratios. Scale bar—50 μm. * $p < 0.05$, $n = 6$ distinct donors

not abrogate lorazepam mediated calcium mobilization [11]. This can be explained in part by recent studies by the Emala and Cook labs that have shown ASM expresses the α4 subunit of the $GABA_A$ receptor [22, 23], which is not responsive to classical benzodiazepines [24]. In this study, we extend our previous work by demonstrating the ability of lorazepam and sulazepam to differentially regulate ASM contractile state in hPCLS, and identifying 2 separate mechanisms mediating the relaxant effects of sulazepam.

In isolated ASM cells subjected to magnetic twisting cytometry (MTC), sulazepam demonstrated superior efficacy and potency relative to that of lorazepam (Fig. 2). In the integrated airway environment (such as PCLS tissues) however, epithelium and possibly other cell types may influence outcomes. Figure 1 data demonstrate similar effects to those observed with MTC for lorazepam and sulazepam regulation of ASM contractile state in hPCLS, suggesting that ASM is the predominant target of OGR1-activating benzodiazepines in the airway. Consistent with our previous study demonstrating the role of PKA activity in effecting relaxation of ASM by G_s-coupled GPCRs such as the β_2AR [20], we herein demonstrate that G_s-biased sulazepam, and not lorazepam, stimulates strong PKA activation as evidenced by the induction of pVASP and pHSP20, and this induction is associated with a decrease in phosphorylation of MLC (a critical effector of ASM contraction). Conversely, lorazepam, by virtue of its "balanced" nature (i.e. ability to activate both G_q and G_s pathways), is able to induce MLC phosphorylation, although unable to affect the induction histamine likely due to histamine and OGR1 receptors competing for the same pool of G_q), which also likely contributes to its inability to inhibit MLC-promoted ASM contraction. Lack of regulation of contraction was further compounded by the fact that we do not see significant activation of PKA substrates by lorazepam in this study, likely due to the media pH utilized in the current experiments (pH 7.6); findings in Pera et al. predict cAMP induction by lorazepam to be low at such a (high) pH [11]. Finally, we further demonstrate the ability of sulazepam to regulate cytoskeleton integrity, which in numerous studies we have shown to be a powerful regulator of ASM contractile state [14, 25, 26].

In summary, the present study demonstrates the qualitative signaling properties of different benzodiazepines can be exploited to manage ASM contractile state. The G_s-biased OGR1 agonist sulazepam, and not the balanced agonist lorazepam, is able to relax contracted ASM in both integrative (hPCLS) and more reductionist (ASM cells in culture) models for assessing ASM contraction. Sulazepam-mediated relaxation of ASM contraction is associated with the superior, and selective, ability of sulazepam to effect G_s/PKA signaling and inhibit MLC phosphorylation, and to regulate cytoskeleton integrity via changes in F/G actin. Ideally, safe and sufficiently selective G_s-biased OGR1 ligands will be ultimately developed and add to our current armamentarium of anti-asthma drugs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-025-03268-9.

Additional file 1

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Authors' contributions
DRV, AKJ, EJ, ID, RW and APN conducted the experiments presented in the manuscript. DRV and APN analyzed the data sets for hPCLS and western blot data, while SSA analyzed MTC data and DDT analyzed actin cytoskeleton reorganization data. RAP provided critical resources of hPCLS and hASM cells. RBP, DAD and APN conceptualized the study and wrote the primary draft of the manuscript. All authors read and approved the final manuscript.

Data availability
Raw and analyzed data files are stored on institutional LabArchives repository and institutional storage drives and will be made available by the corresponding author upon request.

Declarations

Ethics approval and consent to participate
The research activities presented in the manuscript use tissues and cells sourced from human donors. These tissues and cells have been deemed non-human subject research by the Thomas Jefferson University Institutional Review Board (IRB).

Competing interests
Drs. Deshpande and An are Editors of Respiratory Research journal.

Author details
[1]Department of Medicine, Center for Translational Medicine & Division of Pulmonary and Critical Care Medicine; Jane and Leonard Korman Lung Center, Thomas Jefferson University, Philadelphia, PA 19107, USA. [2]Rutgers University, New Brunswick, NJ, USA. [3]Department of Molecular and Cellular Physiology, Albany Medical College, Albany, NY, USA.

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