Novel Small Airway Bronchodilator Responses to Rosiglitazone in Mouse Lung Slices

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Abstract

There is a need to identify novel agents that elicit small airway relaxation when β2-adrenoceptor agonists become ineffective in difficult-to-treat asthma. Because chronic treatment with the synthetic peroxisome proliferator activated receptor (PPAR)γ agonist rosiglitazone (RGZ) inhibits airway hyperresponsiveness in mouse models of allergic airways disease, we tested the hypothesis that RGZ causes acute airway relaxation by measuring changes in small airway size in mouse lung slices. Whereas the β-adrenoceptor agonists albuterol (ALB) and isoproterenol induced partial airway relaxation, RGZ reversed submaximal and maximal contraction to methacholine (MCh) and was similarly effective after precontraction with serotonin or endothelin-1. Concentration-dependent relaxation to RGZ was not altered by the β-adrenoceptor antagonist propranolol and was enhanced by ALB. RGZ-induced relaxation was mimicked by other synthetic PPARγ agonists but not by the putative endogenous agonist 15-deoxy-PGJ2 and was not prevented by the PPARγ antagonist GW9662. To induce airway relaxation, RGZ inhibited the amplitude and frequency of MCh-induced Ca2+ oscillations of airway smooth muscle cells (ASMCs). In addition, RGZ reduced MCh-induced Ca2+ sensitivity of the ASMCs. Collectively, these findings demonstrate that acute bronchodilator responses induced by RGZ are PPARγ independent, additive with ALB, and occur by the inhibition of ASMC Ca2+ signaling and Ca2+ sensitivity. Because RGZ continues to elicit relaxation when β-adrenoceptor agonists have a limited effect, RGZ or related compounds may have potential as bronchodilators for the treatment of difficult asthma.

Keywords: airway smooth muscle; asthma; bronchodilator; lung slices; rosiglitazone

Contraction of small airways contributes significantly to the airflow limitation in asthma (1, 2) and is an important but relatively neglected target for treatment (3, 4). Although β2-adrenoceptor agonists such as albuterol (ALB) are an effective therapy for acute asthma symptoms, isolated small airways from humans (usually defined as < 2 mm diameter) have been shown to be less sensitive to their bronchodilator effects than larger airways (5, 6). This may contribute to an impaired ability of β2-adrenoceptor agonists to reverse small airway contraction in severe asthma (1, 7). Furthermore, the continual or excessive use of β2-adrenoceptor agonists can lead to tachyphylaxis and a loss of response to this class of drugs (8). Therefore, there is a pressing need for novel agents that can elicit relaxation of small airways via alternative pathways to β2-adrenoceptor agonists.

A possible candidate is rosiglitazone (RGZ), a potent synthetic agonist of the nuclear peroxisome proliferator activated receptor (PPAR)γ that is expressed at increased levels in airway smooth muscle cells (ASMCs) in asthma (9). RGZ and ciglitazone (CGZ) regulate ASMC proliferation and the production of...
inflammatory mediators such as GM-CSF in vitro (10). Consistent with these actions, chronic treatment with PPARγ agonists inhibits inflammatory cell influx, airway wall remodeling, and the development of airways hyperresponsiveness to the bronchoconstrictor methacholine (MCh) in mice with ovalbumin-induced airway disease (11–15) (reviewed in Reference 10).

RGZ has also been shown to improve lung function in the absence of detectable antiinflammatory actions in a mouse ovalbumin model (13) and in a small clinical trial that studied smokers with asthma (16). In the latter study, oral treatment with RGZ over 4 weeks produced improvements in forced expiratory flow values as compared with treatment with the inhaled steroid beclometasone dipropionate; this result may reflect a direct effect of RGZ to reduce small airway obstruction (16). In another study, RGZ was shown to relax mouse isolated tracheal preparations precontracted with carbachol (17), but its acute effects on small airways were not assessed.

The major aim of this study was to assess if RGZ and other PPARγ agonists can serve as novel bronchodilators of small airways. To evaluate this potential, mouse lung slices were prepared containing small airways. To evaluate this potential, mouse isolated tracheal tissue was not assessed. (17), but its acute effects on small airways was shown to relax mouse isolated tracheal tissue (16). In another study, RGZ effect of RGZ to reduce small airway obstruction (16). In another study, RGZ was shown to relax mouse isolated tracheal preparations precontracted with carbachol (17), but its acute effects on small airways were not assessed.

Preparation of Lung Slices

All procedures were approved by the Ethics Committees of Melbourne University and the Massachusetts Medical School. Male Balb/C mice (6–12 wk) were killed by an overdose of sodium pentobarbitone. Their lungs were inflated with ~ 1.4 ml of 2% agarose gel dissolved in HBSS supplemented with 20 mM HEPES (sHBSS) followed by ~ 0.5 ml air to push the gel into the alveolar sacs. After solidifying the agarose in sHBSS (4 °C, 20 min), lung slices containing 0.1% Pluronic F-127 (Sigma-Aldrich) were sectioned using a vibratome (VT 1000S, Leica) and cultured in supplemented DMEM (1% penicillin-streptomycin, 37 °C, 5% CO2) as described previously (19, 24).

Mounting and Microscopy

Slices were mounted in a custom-made chamber (~ 100 μl volume) and observed under phase contrast on an inverted microscope (Diaphot 300; Nikon, Melville, NY) using a 10× objective lens, a zoom adaptor, a reducing lens, and a CCD camera (TM-62EX; Pulnix, JAI Inc., San Jose, CA). Single airways (200–400 μm) with an intact epithelium displaying ciliary activity were selected for experimentation.

Image Capture and Analysis

Digital images (744 × 572 pixels) recorded in time lapse (0.5 Hz) using imaging software (Video Savant; IO Industries, London, ON, Canada) were analyzed using NIH/Scion (Scion Corp., Torrance, CA). An appropriate gray-scale threshold distinguished between the airway lumen and surrounding tissue and changes in area with time were calculated by pixel summation.

Lung Slice Perfusion

Individual solutions were delivered over timed intervals using a perfusion control system (Warner Instruments, Hamden, CT) and gravity-powered flow (~ 3 ml/min) (22). Dilator responses were assessed in airways precontracted with MCh, serotonin (5HT), or endothelin (ET)-1. The effects of RGZ on the development of contraction to MCh were also assessed. RGZ responses were measured in the presence of PPARγ antagonist GW9662 (10 μM), propranolol (10 nM), SQ22536 (100 μM), indomethacin (10 μM), the nitric oxide synthase inhibitor No-nitro-L-arginine (NOLA) (100 μM), the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (10 μM), and tetraethylammonium (TEA) (1 mM).

Measurement of Intracellular Ca2+-Slices were loaded with 20 μM Oregon Green-AM in sHBSS (~ 10 slices/ml) containing 0.1% Pluronic F-127 (Sigma-Aldrich, St. Louis, MI) and 100 μM sulforhodamine (1 h; 30°C), followed by sHBSS containing 100 μM sulforhodamine (30 min) (19, 24).

Fluorescence images of ASMCs were observed using confocal or two-photon microscopy as described previously (19, 24) and recorded by VideoSavant (IO Industries, London, ON, Canada) (30 Hz). The average fluorescence intensity of a region of interest (~ 5 × 5 pixels) inside a single SMC was analyzed with Scion image software and custom-written macros. Final fluorescence values (Ft) in response to 300 nM MCh and increasing RGZ were expressed as the ratio (Ft/F0) normalized to the initial fluorescence (F0).

Preparation of Ca2+-Permeabilized Slices

After control responses to 300 nM MCh in the absence and presence of RGZ were obtained, slices were exposed to 20 mM caffeine/50 μM ryanodine (5 min) to clamp intracellular calcium ([Ca2+]i) at a sustained high level (19). Ca2+...
peptidase was confirmed by the absence of caffeine-mediated contraction. Responses due to alterations in Ca$^{2+}$ sensitivity alone were then determined.

Statistics
All data were expressed as mean ± SEM, with each n representing one slice per mouse, for analysis using Graph Pad Prism, with P < 0.05 considered statistically significant. All responses were averaged over the final minute of perfusion. To correct for differences in airway size, contractions were normalized to % initial area. Relaxation responses were expressed as % initial precontraction, with concentration-response curves fitted to obtain half maximal effective concentration (EC$_{50}$) and maxima. RGZ responses in the absence and presence of inhibitors were compared by two-way ANOVA or one-way ANOVA with Dunnett’s post hoc test. Responses to single concentrations of agonists were compared by paired or unpaired $t$ tests as appropriate.

Results
Comparison of RGZ and β-Adrenoceptor Agonists
To assess the ability of RGZ to elicit relaxation relative to β-adrenoceptor agonists, we first precontracted small airways with 300 nM MCh, a concentration that causes submaximal contraction (25). Representative images of a single small airway and frame-by-frame analysis of changes in airway lumen area show that cumulative additions of RGZ reversed the MCh-induced precontraction (Figure 1A). In separate airways precontracted to a similar approximately 40% reduction in lumen area in response to MCh (Figure 1B), RGZ, isoproterenol (ISO), and ALB elicited relaxation (Figure 1C). Their relative potencies were ISO > ALB > RGZ, with RGZ being approximately 100-fold less potent than ISO. However, only RGZ caused near-complete relaxation, with neither ISO nor ALB being able to fully reverse the contraction to MCh (maximum relaxation, 100 μM RGZ, 93.4 ± 2.1%; 100 nM ISO, 66.7 ± 12.6%; 10 μM ALB, 41.2 ± 7.5%; one-way ANOVA, $P < 0.05$) (Figure 1C).

RGZ was also able to inhibit the development of MCh-induced contraction. The reduction in airway lumen area in response to MCh (42.4 ± 4%; n = 4) was prevented in a concentration-dependent manner by RGZ, with no contraction to MCh evident in small airways in the presence of 100 μM RGZ (see Figure E1 in the online supplement).

To confirm that RGZ was not acting via β-adrenoceptors, we assessed RGZ-mediated relaxation in the presence of an effective concentration of propranolol, a β-adrenoceptor antagonist. Propranolol significantly decreased the potency of ISO (EC$_{50}$: control, 17.9 ± 5.1 nM; +propranolol, 270 ± 115 nM; unpaired $t$ test, $P < 0.05$) but not RGZ (EC$_{50}$: 3.5 ± 1.3 μM) (Figure E2).

Additive Effects of RGZ and ALB
Because RGZ was able to cause relaxation independently of β-adrenoceptor activation, we wanted to determine whether it was able to overcome the residual contraction that could not be reversed by ALB (Figure 2). In airways matched for precontraction (Figure 2A), treatment with 10 μM ALB alone elicited 37.7 ± 2.0% relaxation (Figure 2B). In these partially relaxed airways, the effects of RGZ and ALB were additive, and the potency of RGZ was increased approximately 3-fold (EC$_{50}$: RGZ control, 0.39 ± 0.13 μM) (Figure 2B).

Effects of Increasing Contraction on Responses to RGZ and ALB
We then wanted to determine the effect of varying the level of precontraction with MCh on the relative dilator capacities of RGZ and ALB (Figure 3). Before assessment of RGZ-mediated relaxation, MCh at 30, 300, and 3,000 nM caused increasing reductions in airway lumen area by 11.6 ± 4.9%, 39.1 ± 3.0%, and 53.4 ± 1.1%, respectively. Although the maximum relaxation with RGZ was delayed with increasing MCh concentrations, its efficacy was maintained (Figures 3A and 3C).

Figure 1. Rosiglitazone (RGZ), but not β-adrenoceptor agonists, elicits full relaxation of mouse small airways in lung slices. Airways in lung slices were precontracted with 300 nM methacholine (MCh) to achieve ~70% of the maximum MCh response before perfusion with increasing concentrations of RGZ, albuterol (ALB), or isoproterenol (ISO). (A) Representative phase-contrast images of the last frame of each 5-minute perfusion period and frame-by-frame analysis (0.5 Hz) show changes in lumen area with time in the presence of MCh and RGZ. Responses (mean ± SEM) to precontraction with MCh (B) before relaxation with RGZ (n = 4), ALB (n = 8), and ISO (n = 7) (C) are expressed as % initial airway lumen area and % relaxation of the submaximal MCh precontraction, respectively.

Figure 2. Representative images of a single small airway in response to MCh (42.4 ± 4%; n = 4) was prevented in a concentration-dependent manner by RGZ, with no contraction to MCh evident in small airways in the presence of 100 μM RGZ (see Figure E1 in the online supplement).
Relaxation to RGZ against Different Contractile Agonists

Although MCh is most commonly used to assess bronchodilator responses, Et-1 and 5HT also elicit contraction of mouse airways (26). We tested the ability of RGZ to reverse contraction to these contractile agonists using concentrations previously defined to produce submaximal precontraction in mouse small airways (22, 25) (Figure 4A).

The potency and efficacy of RGZ was similar irrespective of the contractile agonist used, with 50% relaxation to RGZ occurring at 45 ± 15, 49 ± 17, and 14 ± 4 μM against MCh, 5-HT, and Et-1, respectively (Figure 4A).

Comparison of RGZ with other PPARγ Agonists

To explore the mechanism underlying the acute bronchodilator response to RGZ, other structurally related PPARγ agonists were assessed. In airways matched for precontraction to 300 nM MCh, RGZ and troglitazone elicited complete relaxation with similar potency (Figure 4B). CGZ was less effective, with only partial relaxation evident up to 100 μM CGZ (Figure 4B). Pioglitazone (PGZ) (30 μM) elicited similar relaxation to 30 μM CGZ (21.9 ± 3.0%; n = 3), but higher concentrations of PGZ were not tested due to its limited solubility.

Because all these glitazones are known agonists of PPARγ, we wanted to determine whether relaxation to RGZ was mediated through PPARγ activation. We used a concentration of the selective PPARγ antagonist GW9662 (2-chloro-5-nitrobenzanilide) that prevented the antiproliferative effects of RGZ in human ASMCS (27). RGZ-mediated relaxation was maintained in the presence of GW9662, suggesting a PPARγ-independent mechanism (Figure 4C). In support of this finding, small airway contraction was not reversed by 100 μM 15-deoxy-D12,14-prostaglandin-J2 (15 d-PGJ2), a putative endogenous PPARγ agonist that is structurally unrelated to the glitazones (% reduction in lumen area to 300 nM MCh: control, 37.4 ± 4.7%; +15 d-PGJ2, 38.1 ± 4.3%; [n = 3]; unpaired t test, NS)

Effect of Inhibitors on Bronchodilator Responses to RGZ

Given that RGZ appeared to cause airway relaxation independently of activation of β-adrenoceptors or PPARγ, we used a variety of pharmacological inhibitors to implicate other mechanisms of action. We focused on the potential contributions of intracellular signaling pathways, of the
We then directed our attention to the ability of RGZ to regulate ASMC Ca\(^{2+}\) signaling and sensitivity. MCh-induced contraction is associated with Ca\(^{2+}\) oscillations within ASMCs due to cycles of Ca\(^{2+}\) release and reuptake from the sarcoplasmic reticulum. We measured the effects of RGZ on Ca\(^{2+}\) oscillations in the presence of 300 nM MCh. The increases in Ca\(^{2+}\) oscillation frequency and amplitude in response to MCh were slightly reduced by 10 μM RGZ, whereas a representative trace shows a marked reduction in the presence of 100 μM RGZ (Figure 5A). Ca\(^{2+}\) oscillations in the absence of RGZ (28.0 ± 5.1 Hz; n = 4) were inhibited by 76.8 ± 8.5% in the presence of 100 μM RGZ (Figure 5B).

MCh-induced contraction is also associated with increased Ca\(^{2+}\) sensitivity, mediated through Rho kinase signaling. This can be studied in isolation using Ca\(^{2+}\)-permeabilized slices in which MCh-induced Ca\(^{2+}\) oscillations are abolished pharmacologically. Before Ca\(^{2+}\) permeabilization, a normal contractile response to 300 nM MCh and the expected near-complete relaxation in response to 100 μM RGZ were observed (Figure 6A). Ca\(^{2+}\) permeabilization with caffeine/ryanodine to lock ryanodine receptors in an open state and clamp [Ca\(^{2+}\)]i levels to a steady state was achieved using ASMCs treated with 100 μM RGZ for 15 min, and subsequent exposure to caffeine did not cause contraction, confirming that [Ca\(^{2+}\)]i levels had been clamped and that subsequent responses could be attributed to altered Ca\(^{2+}\) sensitivity alone.

**Definition of abbreviations**: INDO, indomethacin; NOLA, Nω-nitro-L-arginine; ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; RGZ, rosiglitazone; TEA, tetraethylammonium.

**Table 1**: Effect of Inhibitors on Relaxation to Rosiglitazone in Mouse Small Airways*

<table>
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<th>Inhibitor</th>
<th>Target</th>
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<th>+ Inhibitor + RGZ</th>
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<td>NOLA (100 μM)</td>
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<td>42.3 ± 6.9(^f)</td>
<td>90.9 ± 3.1</td>
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*Airways in lung slices were continuously perfused with 300 nM methacholine to elicit a precontraction before addition of inhibitor alone followed by inhibitor and 100 μM RGZ. Responses (mean ± SEM) are expressed as % relaxation of the submaximal precontraction averaged over the last minute of perfusion as described in MATERIALS AND METHODS.

\(^f\)P < 0.05 (paired t test methacholine precontraction alone).
induced by 300 nM MCh and the inhibitory effect of 100 μM M Ch incubated with 20 μM Oregon Green-AM. (A) Representative trace showing Ca²⁺ oscillations induced by 300 nM MCh and the inhibitory effect of 100 μM RGZ. (B) Ca²⁺ oscillation frequency of ASMCs after 4 minutes of RGZ exposure. Responses (mean ± SEM) are expressed as % of the Ca²⁺ oscillation frequency in the presence of MCh in four to eight slices from four mice. *P < 0.05 one-way ANOVA, methacholine precontraction alone. Fᵢ/F₀ = ratio of final fluorescence normalized to the initial fluorescence.

The magnitude of contraction to MCh was similar within the same airway before and after Ca²⁺ permeabilization, with reductions in airway lumen area of 47.0 ± 5.7% and 43.2 ± 6.3%, respectively (n = 4). When a single addition of 100 μM RGZ was repeated after caffeine/ryanodine treatment, relaxation occurred at a slower rate without reaching a plateau response within 5 minutes (Figures 6A and 6B), and the average % relaxation over the last minute of perfusion decreased from 86.1 ± 1.1% to 70.4 ± 2.9% under Ca²⁺-permeabilized conditions (P < 0.05, paired t test). However, there was no difference in the potency or maximum relaxation to RGZ derived from concentration-relaxation curves prepared under control conditions or after caffeine/ryanodine treatment (not significant, unpaired t tests) (Figure 6C).

Discussion

Although β₂-adrenoceptor agonists are commonly used for the relief of acute asthma symptoms, there is a need for alternative bronchodilators for patients with poorly controlled asthma (33, 34). With repeated use, tolerance to β₂-adrenoceptor agonists can occur. In addition, these drugs may have only limited efficacy in small airways (5, 6), where there is evidence of increased inflammation (35) and airway wall remodeling (36) and higher airway resistance in patients with mild asthma compared with healthy subjects (1, 37). In vitro studies also indicate that small airways are relatively more sensitive to contractile mediators (38).

PPARγ is a widely expressed ligand-activated transcription factor implicated in human lung diseases associated with inflammation and remodeling (10, 14). PPARγ is the target for the thiazolidinedione class of synthetic antidiabetic agents, including RGZ (39), PGZ, and CGZ as well as the proposed endogenous agonist 15 d-PGJ₂. Activation of PPARγ results in suppression of cytokine production from activated macrophages and monocytes, T cells, airway epithelial cells, and ASMCs (10). In human ASMC cultures, we and others have shown that PPARγ ligands also decrease proliferation (14, 27, 40), with inhibition prevented by the PPARγ antagonist GW9662 (27).

In bronchial biopsies from asthmatic human airways, increased PPARγ expression occurs in inflammatory and epithelial cells (9). Consequently, the role of PPARγ has been explored in models of allergic airways disease in mice. Chronic treatment with RGZ and CGZ inhibited the influx of eosinophils and lymphocytes (15), the deposition of collagen and mucus in the airways (11), and inhibition of AHR (13, 14). Here, we explored the potential direct bronchodilator effects of RGZ and other PPARγ agonists in small airways of mouse lung slices. To achieve this, we used the lung slice technique, which offers many advantages for evaluating small airway reactivity in vitro. Critically, a quantitative analysis of changes in airway lumen area and Ca²⁺ signaling within ASMCs can be simultaneously performed during assessment of responses to constrictor and dilator agents (41, 42).

First, we demonstrated acute relaxation to RGZ and the β-adrenoceptor agonists ISO and ALB after submaximal contraction with MCh. RGZ was able to elicit concentration-dependent and near-complete relaxation within minutes, whereas only partial relaxation was evident for either of the β-adrenoceptor agonists. The nonselective β₁/β₂-adrenoceptor agonist ISO was more potent and elicited greater relaxation than the selective β₂-adrenoceptor agonist ALB. This finding can be attributed to ISO being a full agonist and ALB being a partial agonist and is consistent with the contribution of β₁- and β₂-adrenoceptors to mouse airway relaxation (43). Using the nonselective β-adrenoceptor competitive antagonist propranolol, we confirmed that ISO was eliciting relaxation via β-adrenoceptors but that responses to RGZ were unaltered, implicating an alternative mechanism for RGZ.

Given the importance of combination therapy in asthma patients, we assessed the bronchodilator effects of RGZ in the presence of ALB. We found that the effects of RGZ were additive, with the partial relaxation achieved in the presence of a maximally effective concentration of ALB. This finding contrasts with a single study in mouse trachea, where ISO-mediated relaxation was not increased in the presence of RGZ despite a modest dilation seen with RGZ alone (17). However, these contrasting results have been obtained under different experimental conditions, measuring either...
changes in area of small airways in perfused lung slices or changes in force in trachea under static conditions. Differences in airway size, in the potential for accumulation of endogenous relaxing and/or constricting factors depending on incubation time and the technique used, and in the order in which the drugs are added may be important factors in assessing whether there is additivity and/or synergy between RGZ and β-adrenoceptor agonists.

A major limitation of the current therapy in severe asthma is the failure of β-adrenoceptor agonists to fully reverse symptoms. The effectiveness of ALB has been shown to be less effective with increasing levels of MCh-induced tone (44). We wanted to determine if the higher efficacy of RGZ relative to β-adrenoceptor agonists under conditions of submaximal contraction was maintained in maximally contracted airways. Our results clearly demonstrate that RGZ, but not ALB, was able to overcome the increasing functional antagonism in the presence of high concentrations of MCh. This finding suggests that RGZ may be able to reverse severe bronchoconstriction when β-adrenoceptor responsiveness is limited.

Further evidence of the potential of RGZ as a novel bronchodilator was its ability to overcome small airway contraction induced by 5HT or Et-1. Although 5HT is not thought to play a significant role in human asthma, it is a key mast cell–derived mediator in rodents (45). The reversal of Et-1–mediated contraction is of particular interest because it is among the most potent bronchoconstrictors described in human airways (46) and because its levels are increased in asthmatic airways (47).

Because many actions of RGZ are mediated by the nuclear receptor PPARγ, we explored its potential involvement in RGZ-mediated relaxation. Even though other synthetic PPARγ agonists relaxed small airways, the failure of the endogenous PPARγ agonist 15 d-PGJ2 to oppose MCh-induced contraction suggests that an alternative mechanism is more likely to be involved. PPARγ independence was also supported by the finding that relaxation to RGZ was maintained in the presence of GW9662, a PPARγ antagonist (27). Because all glitazones tested were not equally potent, it would be of interest to conduct structure-activity studies to identify key elements associated with dilator potency.

RGZ-induced relaxation was unaffected by the adenylate cyclase inhibitor SQ22536 at a concentration that inhibits relaxation to β-adrenoceptor agonists (28). In addition, dilator responses to RGZ were maintained in the presence of validated effective concentrations of indomethacin or NOLA (17, 31). These findings allowed us to exclude the possible contributions of cAMP or epithelial-derived relaxing factors such as PGE2 or NO to RGZ-mediated relaxation of small airways in perfused lung slices.

A previous study in mouse trachea had shown indomethacin-sensitive relaxation in response to RGZ in a static organ bath under isometric conditions (17). This relaxation was attributed to inhibition of endogenous PGE2 metabolism (48), with PGE2 accumulating at high enough levels to elicit ASM relaxation. However, this mechanism is unlikely to have contributed to RGZ-mediated dilation in the current study because perfusion of lung slices is likely to preclude accumulation of sufficient PGE2 to contribute to small airway relaxation.

We then explored a role for regulation of K+-channel activity in RGZ-induced relaxation. Membrane hyperpolarization resulting from activation of store-operated Ca2+ channels has been proposed as a mechanism for ASM relaxation in response to the recently described novel bronchodilators, the bitter taste agonists (49). However, because relaxation to RGZ was maintained in the presence of TEA, it remains to be determined whether changes in ion conductance contribute to RGZ-induced relaxation.

Finally, we assessed the effects of RGZ on the increases in Ca2+ signaling and sensitivity that underlie airway contraction. We found that, over the same concentration range that elicited airway relaxation, RGZ inhibited the MCh-induced increase in Ca2+ oscillations within ASMCs. The maximum inhibition

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Figure 6. RGZ inhibits MCh-induced increases in calcium sensitivity in mouse small airways. Airways in lung slices were precontracted with 300 nM MCh before the addition of 100 μM RGZ. After washout, slices were perfused with 20 mM caffeine (caff)/50 μM ryanodine (ryano), opening intracellular Ca2+ stores (a transient contraction) and clamping [Ca2+]i levels. A second caffeine application did not evoke contraction, confirming the Ca2+-permeabilized status of the lung slice. Subsequent exposure to 300 nM MCh induced similar contraction due to increased Ca2+-sensitivity alone before assessment of dilator responses to RGZ. (A) Representative trace of responses to 300 nM MCh and 100 μM RGZ under control and Ca2+-permeabilized conditions. (B) Frame-by-frame analysis of responses to 100 μM RGZ in the same airways under control and Ca2+-permeabilized conditions. Data are expressed as mean ± SEM at minute intervals, with data points in between expressed as median only (n = 6). Responses (mean ± SEM) are expressed as % initial airway lumen area (A) and % relaxation of the submaximal precontraction with MCh (B and C).
with RGZ was comparable to that previously reported for ISO in opposing contraction to MCh (24) and greater than that seen in the presence of a maximally effective concentration of ALB (50). This finding is consistent with the relative efficacies of RGZ and the β-adrenoceptor agonists in the current study.

RGZ-induced relaxation was maintained in Ca\(^{2+}\)-permeabilized airways when Ca\(^{2+}\) oscillations had been abolished by pretreatment with caffeine/ryanodine. This indicates that RGZ can reduce MCh-induced increases in ASM C Ca\(^{2+}\) sensitivity. Dual actions on Ca\(^{2+}\) signaling and sensitivity have previously been described for ALB in mouse small airways (50). Like ALB, responses to a maximally effective single concentration of RGZ were delayed after Ca\(^{2+}\) permeabilization (50). It remains to be determined what differentiates the mechanism of action of RGZ from the β-adrenoceptor agonists to enable RGZ to be more effective in mediating small airway relaxation, albeit at lower potency.

Further studies are needed to confirm that the acute bronchodilator effects of RGZ extend to the in vivo setting using animal models of allergic airways disease that mimic the key features of human asthma. In the context of the limited clinical study in which treatment with oral RGZ improved lung function (16), the possibility that inhaled RGZ may elicit acute airway relaxation remains to be tested. However, the current study suggests that PPARγ agonists, including RGZ, may offer therapeutic advantages by exerting control over alternative pathways to β\(_2\)-adrenoceptor agonists.

**Author disclosures** are available with the text of this article at www.atsjournals.org.

**Acknowledgments:** The authors thank Meaghan FitzPatrick, Jean Ni Cheong, Ning Kam, and Mirjam Simoons for technical contributions and Stuart Hirst for valuable discussion.

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