Contractility and Ca^{2+} Signaling of Smooth Muscle Cells in Different Generations of Mouse Airways

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The control and mechanisms of airway smooth muscle cell (SMC) contraction were investigated with a sequential series of lung slices from different generations of the same airway from the cardiac lobe of the mouse lung. Airway contraction was measured by monitoring the changes in airway lumen area with phase-contrast microscopy. Changes in intracellular calcium concentration of the SMCs were studied with a custom-built confocal or two-photon microscope. The distribution of the airway SMCs and the muscarinic M_3 or 5-HT $_{2A}$ receptors was determined with immunofluorescence. Methacholine and 5-HT induced a concentration-dependent airway contraction and Ca^{2+} oscillations within the SMCs of each airway generation. The airway contraction in response to the same agonist concentration was greater in the middle generation compared with the distal or proximal generations of the same airway. Similarly, the Ca^{2+} oscillations varied in different generations of the same airway, with a slower frequency in the SMCs of the distal zone as compared with the middle or proximal zones of airways. By contrast, high KCl induced minimal contraction and very slow Ca^{2+} oscillations throughout the whole intrapulmonary airway. The slower agonist-induced Ca^{2+} oscillations in the distal zone correlated with a reduced expression of agonist receptors. The layer of SMCs increased in thickness in the middle and proximal zones. These results indicate that the contractility of airway SMCs varies at different positions along the same airway and that this response partially results from different Ca^{2+} signaling and the total amount of the SMCs.

Keywords: Ca^{2+} oscillations; two-photon microscopy; lung slices; airway responsiveness

Excessive contraction of airway smooth muscle cells (SMCs) is a major cause of airway narrowing in asthma. This change in the contractile function of SMCs frequently occurs secondarily to airway inflammation, which itself can contribute to increased resistance to airflow by thickening of the airway wall or mucus hypersecretion (1). Although inflammatory responses occur throughout the bronchial tree (2–4), the increased responsiveness and resistance due to airway narrowing appears to be more prevalent in the peripheral airways (1, 5–7). Therefore, to understand this localized reaction, it is important to determine if the contractile responses of SMCs vary in different sections of the respiratory tract and if this predisposes the peripheral airways to disease.

Because of their small size and inaccessible location, the physiology of intact intrapulmonary airways has been difficult to study. As a result, comparative reports of the contractile responses in proximal and distal airways of different species are

CLINICAL RELEVANCE

This study implies that the heterogeneity of airway contraction must be considered when the impact of the hyperreactivity of a diseased airway is evaluated.

inconsistent (8–13). In general, the distal airways appeared more contractile than proximal airways and this difference may result from variations in the structure of the airway wall or the heterogeneity in the SMCs (13). However, a fundamental question is whether the contractility of individual SMC varies in different zones of the intrapulmonary airways. It has been shown that the magnitude of airway SMC contraction relates to the frequency of agonist-induced Ca^{2+} oscillations within the SMCs (14, 15). Consequently, differences in the Ca^{2+} signaling mechanism of SMC (e.g., agonist receptor distribution or type) could be responsible for the variation in contraction at different airway locations.

The aim of this study was to compare the contractile responses of a range of sizes of intrapulmonary airways and correlate these responses with the intracellular Ca^{2+} signaling of the SMCs. The observations of SMC contraction and Ca^{2+} signaling in different airway locations have been made possible by the development of techniques to cut thin serial lung slices that can be observed with microscopy. With this approach, we found that methacholine (MCh) induced a concentration-dependent contraction of SMCs in each generation along the length of an airway through the cardiac lobe of a mouse lung. The greatest contractile response occurred in the midsection of the airway, and this was associated with a high frequency of Ca^{2+} oscillations. A similar variation in the contractile responses and Ca^{2+} signaling of the SMCs was observed at different airway zones in response to another agonist, 5-HT. However, high KCl only elicited a minimal airway contraction and very slow Ca^{2+} oscillations along the whole intrapulmonary airway, a response supporting the idea that membrane depolarization is not a prominent regulatory factor of airway contraction. The thickness of the SMC layer in the airway wall increased significantly from the distal to the middle zone. These results indicate that airway contraction varies with position and that differences in the Ca^{2+} signaling of SMCs and the smooth muscle mass contribute to this variation.

MATERIALS AND METHODS

Most methods have been previously published in detail (14, 15). Only a brief summary is given.

Materials

Cell culture reagents were obtained from GIBCO/Invitrogen Corp (Carlsbad, CA). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO), Calbiochem Inc. (La Jolla, CA), or BD Bioscience (Franklin Lakes, NJ). Hanks' balanced salt solution was supplemented with 20 mM HEPES buffer (sHBSS) and adjusted to pH 7.4. Isotonic high KCl solutions in HBSS were made by adding 50 mM KCl and removing an equivalent amount of NaCl.

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Lung Slices

Male Balb/C inbred mice (7–10 wk old; Charles River Breeding Labs, Needham, MA) were killed by intraperitoneal injection of ~ 0.3 ml (12 ml/kg) of pentobarbital sodium (Nembutal, 50 mg/ml). This procedure was approved by the IACUC of the University of Massachusetts Medical School. The lungs were inflated with ~ 1.3 ml of 2% warm (37°C) agarose through an intratracheal catheter tube and a small bolus of air (~ 0.2 ml) was injected to flush the agarose out of the airway and into the alveoli. The inflated lung lobes were cooled at 4°C until the agarose gelled. The cardiac lobe was sectioned into ~ 140 - μ m serial slices with a vibratome. Sectioning began at the periphery and each slice was inspected until airways, lined with ciliated cells, were detected. Subsequently, each slice was collected and maintained separately in culture media.

Measurement of Airway Contraction

A lung slice, with airways that had a lumen free of agarose, was transferred to a custom-built perfusion chamber consisting of two cover glasses sealed with silicon grease. The slice was held in position by a nylon mesh with a small hole positioned over the selected airway. Different experimental solutions were delivered to the perfusion chamber by gravity-flow under electronic valve control (14). All experiments were performed at room temperature (RT, $20 \pm 2^\circ\text{C}$). Phase-contrast images of airway contraction were collected with an inverted microscope (IX71; Olympus America Inc., Melville, NY) with $\times 10$ objective, zoom-adaptor, and a CCD camera (model LCL-902C; Watec America Corp, Las Vegas, NV) at 1 frame every 2 s. Images were captured by Video Savant, image-acquisition software (IO industries Inc., London, ON, Canada), and analyzed with Scion software and custom-written macros.

Measurement of Ca^{2+} Signaling

To measure intracellular Ca^{2+} signaling, the lung slices were loaded with 20 μM Oregon Green-AM in sHBSS solution (approximately 10 slices in 1 ml) containing 0.1% Pluronic F-127 and 100 μM sulfobromophthalein for 1 h at 30°C, followed by an additional 30 min in sHBSS containing 100 μM sulfobromophthalein for de-esterification of the dye (16, 17). Fluorescence images of airway SMCs were observed as described previously with a custom-built confocal or two-photon microscope (16, 17). Images were recorded by Video Savant software at video rate (30 Hz). The average fluorescence intensity of a region of interest (ROI, $\sim 5 \times 5$ pixels) inside a single SMC was analyzed frame by frame with Scion image software and custom-written macros. Fluorescence values (F) were expressed as the ratio (F/F_0) normalized to the initial fluorescence (F_0).

Immunocytochemistry

Before fixation lung slices were washed twice with PBS. For the immunofluorescence staining of smooth muscle α -actin, the lung slices were fixed with ice-cold 100% acetone for 15 min, washed with PBS (three times for 5 min each), and incubated with 2% BSA (in PBS) for 30 min at room temperature (RT). Subsequently, the slices were incubated with FITC-conjugated mouse monoclonal antibody against smooth muscle α -actin (Sigma-Aldrich; diluted 1:200 in 2% BSA-sHBSS) for 1 h at RT. Slices were washed with PBS ($\times 3$) before fluorescence observation. The fluorescence images were recorded with a two-photon microscope using 800 nm excitation light. A brightfield, nonconfocal image was recorded simultaneously from the transmitted laser light by a second photomultiplier tube. The thickness of the airway SMCs labeled with anti- α actin was expressed by the mean value of the width of fluorescence layer from six evenly distributed points surrounding the airway lumen.

For the immunofluorescence staining of the muscarinic M_3 receptor or the 5-HT_{2A} receptor, the slices were fixed with 4% formaldehyde for 10 min at RT and washed with PBS (three times for 5 min each). The fixed slices were permeabilized with 0.1% Tween 20 (in PBS) for 15 min, washed with PBS (three times for 5 min each), and blocked with 2% BSA (in PBS) for 1 h at RT. Subsequently, the slices were incubated with unconjugated rabbit polyclonal antibody against the muscarinic M_3 receptor (Sigma Aldrich; 10 $\mu\text{g}/\text{ml}$ in 2% BSA-HBSS) or mouse monoclonal antibody against the 5-HT_{2A} receptor (BD Biosci-

ences; 10 $\mu\text{g}/\text{ml}$ in 2% BSA-HBSS) at 4°C overnight. Slices incubated with nonimmunized rabbit or mouse serum (Sigma Aldrich; 0.2% in 2% BSA-HBSS) served as negative controls. The slices were washed with PBS (three times for 10 min each) and incubated with secondary antibody (Molecular Probes; Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG, 10 $\mu\text{g}/\text{ml}$ in 2% BSA-HBSS) at RT for 1 h in darkness. Subsequently, the slices were washed with PBS (three times for 5 min each) and observed with two-photon microscopy. The fluorescence intensity, reflecting the amount of M_3 or 5-HT_{2A} receptors of an individual SMC, was measured by averaging the pixel values of a serial of ROI ($\sim 5 \times 5$ pixels) along the surface of the SMC.

Statistics

Data are expressed as means $\pm$ SEM. Statistical significance was determined by using Student's t test; $P < 0.05$ was considered significant.

RESULTS

For comparison, only the airways from the cardiac lobe of mice were examined. Approximately 25 sequential lung slices, with a thickness of ~ 140 μm , could be collected from each cardiac lung lobe. Within these slices, the course of one or two airways could be followed from the periphery of the lobe to the proximal zone of the lobe. Because it was not possible to follow the airways for their entire length, the exact location of the slice along the respiratory tract, in terms of airway generation related to the trachea, could not be determined. However, when sectioning began at the periphery (moving up the airway), it was relatively straightforward to identify when the initial most distal airway was present. Consequently, the position of the subsequent slices and airways are described in terms relative to the initial peripheral airway.

The first airway generation was defined as the most distal airway which had an intact epithelium containing ciliated cells. Active ciliary beating on the lumen surface was used as an indicator of lung slice viability. The higher-order or next generation of the airway was identified when another airway branch merged into the airway, as described by the "Horsfield order" (18, 19) (Figure 1A). With multiple serial slices, it was possible to follow and identify ~ 7 –10 intrapulmonary generations of the same airway from the cardiac lobe (Figure 1A). The cartilage structures (rings or plates) of the airway wall, typically found in central airways, were not present in these intrapulmonary airways. The mean lumen area of the airways, expressed as a ratio of the lumen area of the first generation (G1), to compensate for variation between mice, increased in a near-linear manner from the distal to the proximal zone (Figure 1B). A representative slice from the middle region of each airway generation was taken for the subsequent experiments. The lumen of all the airways used was devoid of agarose.

Airway Contraction in Response to MCh, 5-HT, and KCl

Upon exposure to a low concentration of MCh (10 nM, data not shown) the airway wall twitched due to the uncoordinated transient contractions of individual SMCs. When the MCh concentration was increased sequentially to 50 nM, 200 nM, and 1 μM , the airway lumen quickly decreased to reach a stable level within 3 min that persisted while the agonist remained present (Figures 2A and 2B). A summary of the extent of airway contraction at these different MCh concentrations was calculated as a percentage of the area before contraction and plotted against airway generation (Figure 2C).

When the airway was treated with 50 nM MCh, the lumen area decreased by $\sim 20\%$ in the fourth- and fifth-generation airways. However, the contractile response of the same airway was attenuated in higher or lower airway generations and ultimately

Figure 1. (A) A series of phase-contrast images of the serial lung slices documenting the change in size of a single intrapulmonary airway along its course from the periphery to the proximal zone of the cardiac lobe of a mouse lung. The position of the airway of interest within the slice is indicated by the *white arrow*. Slices shown were selected from a full sequential series of slices. The relative position of each slice is shown in relationship to the schematic drawing (left of the images) that represents the divergence of the airway from the distal section (first generation, G1) to the proximal section (seventh generation, G7) and by the numbers of slices collected from each generation of the airway. The fourth generation airway transitioned into the fifth generation after merging with another airway (indicated by *horizontal arrow* in the 12th slice). Representative images from at least eight series of sections of one intrapulmonary airway in the cardiac lobes of different mice. (B) The relative mean cross-sectional area of the intrapulmonary airway at various generations in the resting state. The relative area is expressed as a ratio of the area normalized to the area of the first-generation airway (area = $5,677.5 \pm 115.3 \mu\text{m}^2$, corresponding to a diameter of $84.0 \pm 1.1 \mu\text{m}$, assuming a circular shape). From the distal zone (G1, area A1) to the proximal zone (G7, area A7), the lumen area of intrapulmonary airway increased three times ($A7/A1 = 3.09 \pm 0.19$). Each point represents the mean $\pm$ SEM from eight different airways from different cardiac lobes of eight mice.

ditions, the airway contraction at different generations was not altered. At G1, the MCh-induced control contraction was $11.6 \pm 2.9\%$ as compared to $11.5 \pm 1.5\%$ in the presence of inhibitors. Similarly, at G4 and G7 the control contraction was 46.1 ± 3.2 and $20.8 \pm 5.8\%$, whereas the contraction with inhibitors was $45.2 \pm 2.9\%$ and $20.1 \pm 5.1\%$. Each group included four to five different slices from three mice. These results suggest that airway contractility varies with position in intrapulmonary respiratory tract and that this effect is independent of the epithelium.

To clarify whether the heterogeneity of airway contraction was agonist specific, we also compared the contraction of different airway generations induced by 5-HT (100 nM) and a hyperpotassium solution (50 mM K^+). In keeping with our previous findings (14), 5-HT induced a rapid and reversible contraction of airway lumen in all sections of the airway (Figure 3A). The same airways responded to MCh and consistent with the responses to MCh, 5-HT induced the greatest contraction ($\sim 30\%$, Figures 3A and 3B) in G3 airways, while the contractile responses decreased to $\sim 14\%$ in G1 airways and $\sim 19\%$ in G7 airways (Figure 3B). The difference in the lumen contraction between the various generations was greater in response to MCh than 5-HT.

In contrast to MCh and 5-HT, exposure of airways to 50 mM K^+ resulted in little effective contraction; the airway SMCs were observed to twitch asynchronously and in an uncoordinated manner, such that only a minimal luminal contraction ($< 10\%$) occurred. Similar responses were observed in all airway zones from G1 to G7 (Figures 3A and 3B). These results confirmed our earlier results (14) that membrane depolarization induced by hyperpotassium solutions has minimal regulatory effects on intrapulmonary airway contraction.

Ca^{2+} Signaling of Airway SMCs in Response to MCh, 5-HT, and KCl

To explore the mechanisms underlying the difference in the contractile responses of the various generations of intrapulmonary airways, we compared MCh-induced Ca^{2+} signaling in the SMCs of the corresponding airways. Different slices containing G1, G4, or G7 airways were selected and the Ca^{2+} responses of

lost in the most distal generation. When higher concentrations of MCh were applied, larger airway contractions were observed at each airway generation. The greatest contraction ($\sim 40\%$ in response to 200 nM MCh or $\sim 60\%$ in response to 1 μM MCh) occurred within the third (G3) and fourth (G4) airway generation, while a decreasing contraction was observed toward the proximal or distal airways (Figures 2B and 2C).

To determine if this differential airway contraction was a function of factors released by epithelial cells (i.e., NO and prostaglandins), the contractile response to 200 nM MCh was examined after 30 min of exposure to a combination of 100 μM L-NMMA (an inhibitor of nitric oxide synthase) with 10 μM indomethacin (an inhibitor of cyclooxygenase). Under these con-

Figure 2. MCh-induced contractile responses at different generations of the same intrapulmonary airway. (A) A series of phase-contrast images showing the resting state and the maximal contracted state in response to 1 μ M MCh in a distal (G1, first generation), middle (G4, fourth generation), and proximal zone (G7, seventh generation) of the same airway. (B) Representative traces demonstrating the contractile responses of the same airway SMCs at different generations (G1 [light grey line], G4 [black line], G7 [dark grey line]) to successive ascending concentrations of MCh. (C) A summary of the contractility of each generation of the intrapulmonary airway in response to 50 nM (light grey line and solid circles), 200 nM (dark grey line and solid squares) and 1 μ M (black line and solid triangles) MCh. Contraction was calculated after 5 min of agonist exposure. Each point represents the mean $\pm$ SEM from at least five different airways.

Figure 3. The contractile response, induced by 5-HT and KCl, of an intrapulmonary airway at different zones. (A) Representative traces demonstrating the contractile responses of the airway at G1 (light grey line), G4 (dark grey line), and G7 (black line) to a sequential exposure of 100 nM 5-HT, 200 nM MCh, and 50 mM KCl. (B) A summary of the lumen contraction at each airway generation in response to 100 nM 5-HT and 50 mM KCl. Contraction was calculated after 5 min of agonist or KCl exposure. Each point represents the mean $\pm$ SEM from five to eight different airways.

SMCs to MCh, 5-HT, and 50 mM K^+ were measured. In general, upon exposure to MCh or 5-HT, the SMCs from all of three zones responded with an initial increase in $[Ca^{2+}]_i$ followed by oscillations in Ca^{2+} (Figures 4 and 5). These Ca^{2+} oscillations were asynchronous between the adjacent cells. However, the exact frequency of Ca^{2+} oscillations varied in different airway generations in response to different agonists.

When a low concentration of MCh (50 nM) was used, no changes in $[Ca^{2+}]_i$ were observed in the most distal (G1) airways, but slow Ca^{2+} oscillations ($\sim 6 \text{ min}^{-1}$) were detected in the middle or proximal airways. These responses correlated with the absence of a contractile response in G1 airways and a small contraction in G4/G7 airways.

In response to 200 nM MCh, the Ca^{2+} oscillation frequency was significantly increased in the SMCs of all airway generations. This correlates with the overall increased contractility. However, the frequency rate was smaller (G1, $8.0 \pm 1.0 \text{ min}^{-1}$) in the distal airways compared with the Ca^{2+} oscillations of the airway of the middle zone (G4, $14.5 \pm 0.6 \text{ min}^{-1}$) or proximal zone (G7, $16.5 \pm 1.4 \text{ min}^{-1}$, $P < 0.05$) (Figure 4).

When high concentrations of MCh (1 μ M) were applied, the Ca^{2+} responses of SMCs in each airway generation were further increased; the frequency of the Ca^{2+} oscillation were significantly increased in the distal ($13.2 \pm 1.2 \text{ min}^{-1}$) and middle ($18.5 \pm 1.2 \text{ min}^{-1}$) zones compared with the response to 200 nM MCh

($P < 0.05$). The frequency of the Ca^{2+} oscillations was $17.5 \pm 1.3 \text{ min}^{-1}$ in the proximal zone. These results also indicate that the Ca^{2+} response of SMCs to MCh varies with location (Figure 4).

A similar variation in the 5-HT-induced (100 nM) Ca^{2+} oscillations of SMCs existed at different airway zones. The frequency rate was $10.6 \pm 0.8 \text{ min}^{-1}$ in the distal airways (G1), but increased to $\sim 14 \text{ min}^{-1}$ in the mid and proximal airways (G4, $13.8 \pm 1.1 \text{ min}^{-1}$; G7, $13.8 \pm 0.6 \text{ min}^{-1}$; Figure 5). However, exposure to 50 mM K^{+} induced a slight elevation of the baseline fluorescence upon which was superimposed a series of very slow, prolonged and large Ca^{2+} oscillations in all airway zones. The frequency of these oscillations was $1.5 \pm 0.4 \text{ min}^{-1}$ at G1, $2.2 \pm 0.2 \text{ min}^{-1}$

at G4, and $2.4 \pm 0.4 \text{ min}^{-1}$ at G7. In previous studies, we have demonstrated that the mechanism underlying KCl-induced Ca^{2+} oscillations is substantially different from that mediating agonist-induced Ca^{2+} oscillations; KCl-induced oscillations primarily depend on Ca^{2+} influx and over-loading of internal stores whereas agonist-induced oscillations primarily rely on controlled Ca^{2+} release from internal stores (14).

Figure 4. The Ca^{2+} signaling induced by MCh in airway SMCs from different zones of intrapulmonary airways. (A) A representative trace of the intracellular Ca^{2+} signaling of SMCs located within the fourth generation in response to 200 nM MCh. (B) The MCh (50 nM, 200 nM, 1 μM) induced Ca^{2+} signaling of SMCs from distal (G1), middle (G4) and proximal (G7) zones of the airways. Ca^{2+} responses of the same SMC to different concentration of MCh were recorded after 5 min of agonist exposure. The slices were stimulated with ascending concentrations of MCh. In each generation, higher concentrations of MCh induced faster Ca^{2+} oscillations. However, the frequency of the Ca^{2+} oscillations of SMCs in G1 airways was slower than those in G4 or G7 airways in response to the same concentration of MCh. (C) The summary of the frequency of the Ca^{2+} oscillation induced by increasing concentrations of MCh at different generations of the airway. Each column represents the mean $\pm$ SEM from different SMCs from five slices of five different mice. * $P < 0.05$ compared with the frequency of Ca^{2+} oscillations induced by MCh (200 nM) at G4 or G7. # $P < 0.05$ compared with the frequency of Ca^{2+} oscillations induced by MCh (1 μM) at G4.

at G4, and $2.4 \pm 0.4 \text{ min}^{-1}$ at G7. In previous studies, we have demonstrated that the mechanism underlying KCl-induced Ca^{2+} oscillations is substantially different from that mediating agonist-induced Ca^{2+} oscillations; KCl-induced oscillations primarily depend on Ca^{2+} influx and over-loading of internal stores whereas agonist-induced oscillations primarily rely on controlled Ca^{2+} release from internal stores (14).

The comparison of airway contractility with the Ca^{2+} oscillations occurring in the SMCs at each airway generation (for MCh see Figures 2 and 4; for 5-HT see Figures 3 and 5) indicates that the extent of airway contraction was correlated with the frequency of the Ca^{2+} oscillations. This relationship has been extensively investigated in previous studies with mid-sized airways (14, 16). However, in this study we found that the gradient of this correlation varied with airway generation (Figure 6). In the proximal and distal airways, Ca^{2+} oscillations ranging from 5–13 min^{-1} (induced by 50 nM to 1 μM MCh) induced a relatively small airway contraction. By contrast, a similar frequency range of Ca^{2+} oscillation (5–20 min^{-1}) induced a substantially greater airway contraction in the mid-zone airways (Figure 6). The low frequency Ca^{2+} oscillations induced by KCl did not sustain a persistent airway contraction but instead induced uncoordinated transient twitching of individual SMCs. These results indicate that while contraction is related to the frequency of the Ca^{2+} oscillations, other factors decrease the extent of contraction in the proximal or distal zones of the same airway.

Expression of Muscarinic M_3 and 5-HT_{2A} Receptors

To address the mechanisms underlying the differences in the Ca^{2+} oscillations of individual SMC at different airway zones, we examined the distribution of agonist receptors on the SMCs at the G1, G4, and G7 airway generation. From the various receptor subtypes, we initially choose to examine the muscarinic M_3 receptor and the 5-HT_{2A} receptor because these receptors are coupled to intracellular signaling pathways leading to the production of IP₃ through activation of PLC β and have been identified, by previous studies, to initiate Ca^{2+} oscillations of airway SMCs (14, 20). Immunofluorescence staining, correlated with brightfield images, suggested that the M_3 and 5-HT_{2A} receptors were located primarily on the surface of the SMCs surrounding the airway lumen (Figures 7A and 7B). By comparing the fluorescence intensity of different airway generations, we found that the SMC fluorescence in the distal airway zone (G1: M_3 receptor 70.8 ± 3.5 , 5-HT_{2A} receptor 59.9 ± 1.8) was generally weaker than in the middle (G4: M_3 receptor 93.5 ± 5.6 , 5-HT_{2A}

Figure 5. The Ca^{2+} signaling induced by 5-HT and KCl at different generations of the intrapulmonary airways. (A) The representative traces showing the Ca^{2+} signaling of SMCs induced by 100 nM 5-HT (after 5 min of exposure) or 50 mM K^+ (after 4 min of exposure) at G1, G4, and G7. The recording time of response to 50 mM K^+ was extended over 120 seconds due to the slow frequency of the Ca^{2+} oscillations. (C) The summary of the frequency of the Ca^{2+} oscillation induced by 100 nM 5-HT and 50 mM K^+ at G1, G4, and G7. Each column represents the mean $\pm$ SEM from different SMCs from 5–10 airways of four different mice. * $P < 0.05$, comparing the frequency of the Ca^{2+} oscillations between G1 and G4 or G7.

receptor 65.4 ± 2.7) or proximal (G7: M_3 receptor 85.3 ± 4.3 , 5-HT_{2A} receptor 65.1 ± 2.5) zones for either type of receptor. However, the fluorescence differences between the G1 and G4 or G7 were only significantly different for the M_3 receptors. These results indicate that the heterogeneity of receptor density related to the airway zones may contribute to agonist-induced variations in Ca^{2+} signaling and contraction of SMCs.

Thickness of SMCs in Different Airway Zones

Airway contraction is mediated by the force produced by the total muscle mass surrounding the airway. Consequently, the number of SMCs and the force generated by each individual SMC are important factors determining the extent of contrac-

Figure 6. Relationship between the Ca^{2+} oscillation frequency and airway contraction induced by MCh at different airway zones. Data from the concentration-response curve of the airway contraction (Figure 2C, change in airway area) were re-plotted with respect to the corresponding frequency of Ca^{2+} oscillations in response to various concentrations of MCh (Figure 4C). Greater contraction was observed at the same frequency of Ca^{2+} oscillations in the middle (G4) zone compared with the distal or proximal (G1 or G7) zones.

tion. In the present study, we quantified the thickness of the airway SMCs layer by measuring the specific immunofluorescence resulting from anti- α -actin antibodies (Figure 7C). Compared with the thickness of the SMC layer in the distal airways (G1, $5.1 \pm 0.8 \mu\text{m}$), the SMC layer was significantly thicker in the middle (G4, $8.7 \pm 1.0 \mu\text{m}$) or proximal (G7, $10.2 \pm 0.7 \mu\text{m}$) airway zone (Figure 7D). The increased thickness of the SMC layer is consistent with the increased airway contraction of the middle and proximal airways.

DISCUSSION

In this study, we found a variation in the agonist-induced contraction in different generations of a single airway and explored the possibility that differences in the Ca^{2+} signaling within SMCs contributed to the variable contractile responses. The use of serial thin lung slices was especially suited to this study, for several reasons. Importantly, the *in vivo* morphologic and structural characteristics of the airways are retained within each slice, making it possible to follow the entire course of an individual intrapulmonary airway from the periphery to a proximal location. As a result, the contractile response of the airway, complete with the influence of the local tethering, and Ca^{2+} signaling of the responsible SMCs at different airway locations can be compared. This is, to our knowledge, the first time that such a comparative study of size-dependent airway contraction and SMC Ca^{2+} signaling has been documented.

For comparison, we selected, based on location, the same airway from the cardiac lobe for each group of experiments. This airway had a total of 7–10 intrapulmonary generations between the most peripheral and proximal positions observed. However, the main bronchus and the first few bronchiole branches of the cardiac lobe were not included in this study because these structures were either not available or conserved in an adequate condition in our lung slices. We compared the contraction of each airway generation, by examining slices from the central zone of the each generation, in response to either a series of concentrations of MCh, a single concentration of a second agonist 5-HT or membrane depolarization with 50 mM K^+ . We found that, at all concentrations of MCh or 100 nM 5-HT, the

Figure 7. Assessment of the distribution of the muscarinic M_3 and 5-HT $_{2A}$ receptors on SMCs and the thickness of the SMC layer by immunofluorescence. (A) Muscarinic M_3 receptor or (B) 5-HT $_{2A}$ receptor staining from G1, G4, and G7 airways (*upper panels*). The control conditions substituted normal rabbit (control for M_3 receptor) or mouse serum (control for 5-HT $_{2A}$ receptor) for specific antibodies. The corresponding nonconfocal, transmitted-light image for each fluorescence image is shown in the *lower panel*. EC, epithelial cells; L, Lumen. (C) The thickness of airway SMCs surrounding an airway was visualized by immunofluorescence of SMC α -actin (*left, top*) and transmitted-light (*left, bottom*). (D) A summary of the thickness of the SMC layer (equal to the thickness of fluorescence zone) at G1, G4, and G7. Each *column* represents mean $\pm$ SEM from at least five different airways from three mice. * $P < 0.05$, comparing the thickness of SMCs at G1 to that at G4 or G7.

intermediate-sized airways (third to fifth generation) had the greatest contractile responses, whereas the most peripheral (smallest) or proximal (largest) airways had the least contractile response.

These results are consistent with previous studies in canine (21) and porcine airway (22). However, from other studies with

rat lung slices, it was suggested that the contraction of the small airways (in response to MCh or thromboxane) was the most sensitive compared with medium or large airways (11, 23). Unfortunately, differences in the size of experimental animal and the method of airway classification confound the comparison. Instead of performing a relative comparison of different generations of the same airway, the average responses of airways of a similar size were compared. In the rat, the small airways were defined to have a diameter of $< 250 \mu\text{m}$; this range includes most of the airways (small to large) examined in this study of mouse airways.

While it has been found that the extent of agonist-induced contraction is influenced by airway size, the differences in the species of interest, the research methods used, and the size of the airways investigated have resulted in a number of hypotheses that account for variation in airway contractility. These include differences in the cellular characteristics of individual SMC, the mass of SMCs, or the elastic resistance of the airway wall that opposes contraction. For example, several studies have demonstrated differences in the receptor density or receptor types on the surface of SMCs (24–27), the ratio of SMCs to the airway area (28–30), the stiffness of airway wall (31, 32), and orientation of the SMCs (29, 30, 33, 34). In addition, the airways may also be influenced differentially by the epithelia-derived relaxing factors (35, 36). With respect to this last point, we found that preincubation of the lung slice with a combination of $100 \mu\text{M}$ L-NMMA and $10 \mu\text{M}$ indomethacin to block the production of both nitric oxide and prostaglandins did not significantly change the MCh-induced airway contraction from the distal to proximal zones. This suggests that the relaxation factors released from the epithelium do not mediate the spatial variation in airway contraction.

Although we could not test all possibilities in one study, we initially addressed this complex problem by refining the use of serial lung slices to provide the ability to perform the necessary comparative studies of the same airway at different generations. Furthermore, the cellular resolution afforded by lung slices allows for the simultaneous correlation of the cellular characteristics of the SMCs with the contraction of the airway. Consequently, we examined if differences in the fundamental mechanism of contraction, namely agonist-induced Ca^{2+} signaling of individual SMC, were responsible for variations in airway contraction.

To simplify the comparison of the Ca^{2+} signaling, SMCs from the first, fourth, and seventh airway generations, which displayed different contractile responses, were selected for examination. Our results demonstrated that MCh and 5-HT induced Ca^{2+} oscillations in the SMCs of all the contracted airways. At each generation, higher concentrations of agonist induced faster Ca^{2+} oscillation and a corresponding greater airway contraction. However, the frequency of the Ca^{2+} oscillations varied with airway generation at all agonist concentrations. The slowest frequency of Ca^{2+} oscillations occurred within the SMCs of the first generation airway and this correlated with the smallest contractile responses. Therefore, the attenuated Ca^{2+} responses and resulting contractility of individual SMC contribute to the weak airway contraction in the distal zone.

A possible reason for slow Ca^{2+} oscillations is that the SMCs have fewer agonist receptors. With quantitative receptor binding analysis, Cheng and Townley (26) reported a significantly higher density of muscarinic receptors in the tracheal SMCs compared with the peripheral lung tissue. ten Berge and coworkers (27) also reported a nonuniform distribution of inhibitory muscarinic M_2 receptors on postganglionic cholinergic nerve endings in different human airways. In this study, we found that the expression of the M_3 receptor, the principal subtype of muscarinic receptor

mediating the IP_3 -induced Ca^{2+} signaling, was also significantly lower in the distal airway zone, a result consistent with the reduced frequency of the Ca^{2+} oscillations in the distal airway zone. The distribution of 5-HT_{2A} receptors did not appear to be substantially different in the various airway generations, even though there was a relatively small variation in the 5-HT-induced Ca^{2+} signaling in different airway zones. Failure to correlate the density of 5-HT_{2A} receptor with the extent of Ca^{2+} signaling may be due to a contribution by other subtypes of 5-HT receptors to the airway contraction, or the relative insensitivity of immunofluorescence to quantify small differences in receptor density.

At all concentrations, the frequency of the Ca^{2+} oscillations in the fourth- and seventh-generation airway SMCs was faster than in the first-generation airways. This increased Ca^{2+} oscillation frequency correlated with a greater extent of airway contraction in the fourth generation. However, in response to similar Ca^{2+} signaling, the contractile response of the seventh generation of airway was always less than the fourth generation of the same airway, a result indicating the existence of additional regulatory factors. While we found that the thickness of the SMC layer increased slightly from G4 to G7 airways, we also found that the size of the airway increased by more than 60% (lumen area). Consequently, the decreased contractile response of the larger airways may arise because of a decrease in total contractile force as a result of the disproportionate increase in SMC mass, relative to the airway diameter (28–30). Alternatively, this response could be explained by an increase in the resistant forces associated with the larger airway.

We also confirmed in this study our previous results (14) that high KCl, commonly believed to induce SMC contraction by stimulating membrane depolarization and Ca^{2+} influx to elevate $[Ca^{2+}]_i$, did not stimulate a sustained airway contraction. By contrast, KCl induced uncoordinated twitching of individual SMCs. Furthermore, there was no substantial variation in this response to KCl throughout the various zones of the airway. These results appear to be inconsistent with the robust contractions induced by high KCl in the tracheal or bronchial SMCs from mice or other species (37, 38). However, we found that the SMC twitching correlated with the occurrence of slow Ca^{2+} oscillations within the individual SMCs rather than sustained elevated levels of Ca^{2+} . Consequently, the larger numbers of SMCs contributing to the contraction of a tracheal ring or muscle strip increases the chance that the responses of these individual SMCs may integrate into a more uniform tissue response. The small contractile response of intrapulmonary airway SMCs to the high KCl may also result from an intrinsic lower Ca^{2+} sensitivity in the absence of agonist (17). Alternatively, the physiology of trachea SMCs may be different from peripheral SMCs. For example, K^+ channels in SMCs from the main human bronchus differ from those in the intralobular airways (39). Therefore it will be necessary to further investigate the variation of contraction and Ca^{2+} signaling in tracheal and bronchial SMCs.

In summary, we have demonstrated with thin lung slices that agonist-induced contraction of SMCs varies along the length of intrapulmonary airway. This can be attributed in part to differences in the Ca^{2+} response of the SMCs of the smaller airways. However, in larger airways with similar Ca^{2+} signaling in SMCs, airway contraction can be modified by the relative mass of airway SMCs. These data emphasize that the heterogeneity of healthy airways must be considered when investigating the basis of hyperreactivity of diseased airways.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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