The Frequency of Calcium Oscillations Induced by 5-HT, ACH, and KCl Determine the Contraction of Smooth Muscle Cells of Intrapulmonary Bronchioles

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ABSTRACT

Increased resistance of airways or blood vessels within the lung is associated with asthma or pulmonary hypertension and results from contraction of smooth muscle cells (SMCs). To study the mechanisms regulating these contractions, we developed a mouse lung slice preparation containing bronchioles and arterioles and used phase-contrast and confocal microscopy to correlate the contractile responses with changes in [Ca\(^{2+}\)], of the SMCs. The airways are the focus of this study. The agonists, 5-hydroxytryptamine (5-HT) and acetylcholine (ACH) induced a concentration-dependent contraction of the airways. High concentrations of KCl induced twitching of the airway SMCs but had little effect on airway size. 5-HT and ACH induced asynchronous oscillations in [Ca\(^{2+}\)], that propagated as Ca\(^{2+}\) waves within the airway SMCs. The frequency of the Ca\(^{2+}\) oscillations was dependent on the agonist concentration and correlated with the extent of sustained airway contraction. In the absence of extracellular Ca\(^{2+}\) or in the presence of Ni\(^{2+}\), the frequency of the Ca\(^{2+}\) oscillations declined and the airway relaxed. By contrast, KCl induced low frequency Ca\(^{2+}\) oscillations that were associated with SMC twitching. Each KCl-induced Ca\(^{2+}\) oscillation consisted of a large Ca\(^{2+}\) wave that was preceded by multiple localized Ca\(^{2+}\) transients. KCl-induced responses were resistant to neurotransmitter blockers but were abolished by Ni\(^{2+}\) or nifedipine and the absence of extracellular Ca\(^{2+}\). Caffeine abolished the contractile effects of 5-HT, ACH, and KCl. These results indicate that (a) 5-HT and ACH induce airway SMC contraction by initiating Ca\(^{2+}\) oscillations, (b) KCl induces Ca\(^{2+}\) transients and twitching by overloading and releasing Ca\(^{2+}\) from intracellular stores, (c) a sustained, Ni\(^{2+}\)-sensitive, influx of Ca\(^{2+}\) mediates the refilling of stores to maintain Ca\(^{2+}\) oscillations and, in turn, SMC contraction, and (d) the magnitude of sustained airway SMC contraction is regulated by the frequency of Ca\(^{2+}\) oscillations.

KEY WORDS: laser scanning confocal microscopy • asthma • airways • arterioles • mouse • lung slices

INTRODUCTION

Gas exchange in the lungs requires an appropriate matching of ventilation to blood perfusion and this is influenced by the caliber of the airways and blood vessels. Consequently, an understanding of the mechanisms that control the size of airways and arterioles is required to understand lung physiology and the development of obstructive lung disease and pulmonary hypertension. In general, the mechanisms that control the caliber of intrapulmonary airways or arterioles have been investigated in either whole lungs or isolated smooth muscle cells (SMCs). While these approaches provide valuable data, it is difficult to determine the site or size of airway or arteriole contraction by measurements of air flow in whole lungs or blood pressure in the pulmonary artery. Similarly, it is difficult to relate changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) of isolated SMCs to the contractile responses of intact airways and arterioles.

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Our solution to investigate how cellular physiology regulates the contraction of the small airways or arterioles was to examine living lung slices that retain many structural and functional properties of the lung. Relatively thick lung slices have been used to study the contractile response of airways (Dandurand et al., 1993; Martin et al., 1996; Minshall et al., 1997; Adler et al., 1998; Duguet et al., 2001; Martin et al., 2001; Wohlsen et al., 2001), but thinner lung slices, combined with confocal microscopy, provided us with the ability to study changes in [Ca\(^{2+}\)] of SMCs that underlie airway contraction (Bergner and Sanderson, 2002a,b, 2003). A subsequent study examined [Ca\(^{2+}\)] in strips of tracheal muscle but these do not preserve the lung architecture or address the physiology of small airways (Kuo et al., 2003). To also examine the equally important role of blood vessels in lung physiology, we modified the lung slice preparation to preserve the structure and function of the arterioles. Although we studied the contraction

Abbreviations used in this paper: ACH, acetylcholine; CPA, cyclopiazonic acid; 5-HT, 5-hydroxytryptamine; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_R\), IP\(_3\) receptor; RyR, ryanodine receptor; SMC, smooth muscle cell.
and relaxation of both the airways and arterioles simultaneously, we focus here, for clarity, on the responses of the airways. We compare the accompanying responses of the arterioles in a second paper (Perez and Sanderson, 2005).

Although 5-hydroxytryptamine (5-HT) is released by neuroendocrine cells in the airway mucosa of animals and humans (Lauwereyns et al., 1973; Lommel, 2001; Adriaensen et al., 2003) the role of 5-HT in airway tone is not well understood. In mice, rats, and guinea pigs, 5-HT induced airway contraction, either in vivo, in isolated lungs, or in isolated trachea rings (Levitt and Mitzner, 1989; Eum et al., 1999; Fernandez et al., 1999; Held et al., 1999; Moffatt et al., 2004). However, this contraction seemed sensitive to atropine and 5-HT was thought to act via a muscarinic pathway. In humans, inhaled 5-HT did not produce bronchoconstriction (Raffestin et al., 1985; Cusshon et al., 1986) but could facilitate cholinergic-induced bronchoconstriction (Dupont et al., 1999). In asthmatics, plasma levels of 5-HT are elevated and correlate with clinical severity (Dupont et al., 1999). In asthmatics, plasma levels of 5-HT are elevated and correlate with clinical severity (Dupont et al., 1999). In asthmatics, plasma levels of 5-HT are elevated and correlate with clinical severity (Dupont et al., 1999). In asthmatics, plasma levels of 5-HT are elevated and correlate with clinical severity (Dupont et al., 1999). In asthmatics, plasma levels of 5-HT are elevated and correlate with clinical severity (Dupont et al., 1999).

An unresolved aspect of airway SMC contractility is the role of membrane depolarization and the influx of extracellular Ca$^{2+}$ (Janssen, 2002). Because contraction induced by either KCl or agonists appears to require extracellular Ca$^{2+}$ (Li et al., 2003), it is believed that a sustained Ca$^{2+}$ influx via voltage-, store-, or receptor-operated channels mediates contraction. However, sustained elevations of Ca$^{2+}$ are often toxic to many cell types including SMCs. Consequently, we re-examined the mechanisms of agonist-induced (5-HT or acetylcholine [ACH]) contraction and voltage-depending (KCl) contraction. We found that 5-HT and ACH induced Ca$^{2+}$ oscillations in airway SMCs with a frequency that determines the size of the airway contraction. By contrast, KCl induced low frequency Ca$^{2+}$ oscillations that induced twitching in airway SMCs. Although both mechanisms required an influx of Ca$^{2+}$, this was found to be primarily required to refill internal stores to maintain the Ca$^{2+}$ oscillations that mediated contraction.

**MATERIALS AND METHODS**

**Materials**

Cell culture reagents were obtained from Invitrogen and GIBCO BRL. Other reagents were obtained from Sigma-Aldrich. Hanks’ balanced salt solution (GIBCO BRL) was supplemented with 20 mM HEPES buffer (sHBSS) and adjusted to pH 7.4. K$^+$/sHBSS was prepared by replacing the sodium salts of sHBSS with potassium counterparts. High KCl isotonic solutions were prepared by mixing K$^+$/sHBSS with normal sHBSS to obtain the desired concentration in K$. Hanks’ “Q” Ca$^{2+}$ solution was prepared by supplementing sHBSS without Ca$^{2+}$ and Mg$^{2+}$ with 0.9 mM MgSO$_4$ and 1 mM of Na$_2$H$_2$-EGTA.

**Lung Slices**

To preserve the normal morphology and study the physiological response of intrapulmonary airways and blood vessels, we modified the preparation of lung slices (Bergner and Sanderson, 2002a). Male BALB/C inbred mice (Charles River Breeding Labs, Needham, MA), between 7 and 9 wk old, were killed by intraperitoneal injection of 0.3 ml of pentobarbital sodium (Nembutal) as approved by the IACUC of the University of Massachusetts Medical School. The trachea was cannulated with an intravascular (IV) catheter tube with two input ports (20G Intima; Becton Dickinson) and secured with suture thread (Dexon II, 4-0; Davis and Geck) to ensure a good seal. A syringe filled with 3 ml of air was attached to one port while the other port was closed. The chest cavity was opened by cutting along the sternum and the ribs adjacent to the diaphragm. To reduce the intrapulmonary blood vessel resistance and facilitate vessel perfusion with gelatin, the collapsed lungs were gently reinfated to approximate their total lung capacity by injecting ~1.5 ml of air. A warm (37°C) solution of gelatin (type A, porcine skin, 300 bloom, 6% in sHBSS) was perfused through the intrapulmonary blood vessels, via the pulmonary artery, by inserting the hypodermic needle of an infusion set (SV x S25BL; Terumo Corporation) into the right ventricle of the heart and slowly injecting ~1 ml of gelatin solution. A small cotton-wool swab soaked in ice-cold sHBSS was placed only on the heart to solidify the gelatin before the perfusion needle was removed. The lungs were deflated by releasing the positive air pressure. A syringe filled with a warm (37°C) solution of 2% agarose (type VII or VII-A; low gelling temperature) in sHBSS was attached to the second port of the catheter. The IV tube was clamped proximal to the trachea and purged of air with the agarose solution by allowing the trapped air to escape via a 27G needle inserted into the IV tube proximal to the clamp. The IV clamp was removed and the lungs were reinfated by injecting ~1.3 ml of agarose-sHBSS. Subsequently, ~0.2 ml of air was injected into the airways to flush the agarose-sHBSS out of the airways and into the distal alveolar space. Immediately after agarose inflation, the lungs were washed with ice-cold sHBSS, and the animal was placed at 4°C for 15 min. The lung and heart were removed and placed in sHBSS (4°C) and cooled for an additional 30 min to ensure the complete gelling of the gelatin and agarose.

To cut thin lung slices, a single lung lobe was removed from the respiratory tree by cutting the main bronchus. The lung lobe was trimmed near the bronchus to produce a flat surface that was adhered to the mounting block of a vibratome (model EMS-4000; Electron Microscope Sciences) using cyanoacrylate glue (Krazy glue, Aron α; Electron Microscope Sciences). The mounted lobe was submerged in a bath of sHBSS maintained at ~4°C. The lung lobe was sectioned into slices ~130 μm thick starting at the lung periphery. The initial slices consisted mainly of agarose-filled alveoli and respiratory bronchioles, but as slicing advanced deeper into the lobe, small arterioles and airways start to appear. Airways were readily identified by their lining of epithelial cells with beating cilia. Serial sections with these features were collected and transferred individually to wells of a 24-well plate containing DMEM supplemented with antibiotics and anti-mycotics and NaHCO$_3$. Fetal serum was not added because it can contain 5-HT (3-5 μM when used at 10%) that can stimulate contraction of SMCs (Abdullah et al., 1994). Slices were kept in culture media at 37°C and 10% CO$_2$ for up to 3 d. At 37°C, the gelatin in the blood vessel lumen dissolved, leaving the blood vessel lumen empty.

**Measurement of the Contractile Response of Airways and Arterioles Induced by Agonists**

Lung slices containing at least one airway and an accompanying arteriole that were cut to reveal a transverse section were se-
lected. Care was taken to ensure that the airways were lined with epithelial cells showing ciliary activity and that the blood vessel wall was intact and not collapsed. It was not uncommon to find blood vessels with elements of their walls separated from the lung parenchyma; these vessels were not used. Slices were transferred to a custom-made perfusion chamber consisting of a Plexiglas support for a 45 × 50 mm coverglass. A lung slice was placed in the center of the coverglass and held in place with a small sheet of nylon mesh (210 μm opening; Small Parts Inc.). To ensure the nylon mesh did not influence the contractile responses of the airways and arterioles or interfere with the phase-contrast optics, a small hole was cut in the mesh, and this was centered over the selected airway and arteriole. A second custom-cut (11 × 40 mm) coverglass edged with silicone grease (Valpey Sealant; Dow Corning Co.) was placed over the slice and nylon mesh.

Perfusion of the lung slice in the chamber was performed by applying a gravity-fed flow of solution at one end of the glass chamber and suction at the other end of the chamber. The volume of the chamber was ~100 μl with a perfusion rate of 800 μl/min. The application of different solutions was controlled by using a custom-built perfusion system consisting of eight solution reservoirs (30 ml) connected to a manifold with a single output way and arteriole lumen was calculated, with respect to time, by distinguishing the lumen from the surrounding tissue, the area of the airways and arterioles of the chamber was described above.

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A stock solution of 2 mM Oregon green 488 BAPTA-AM (Molecular Probes) was prepared with 20 μl anhydrous DMSO and 50 μg Oregon green-AM. An equal volume (20 μl) of 20% Pluronic F-127 (Molecular Probes) in anhydrous DMSO was mixed with the stock solution and diluted in 2 ml of sHBSS containing 100 μM sulfobromophthalein (an inhibitor used to prevent dye extrusion through anion exchangers) to produce a final loading solution of 20 μM Oregon green-AM, 0.2% pluronic, and 100 μM sulfobromophthalein. Slices were incubated for 30 min at 30°C followed by 30 min at room temperature in the loading solution, followed by an additional hour at room temperature in sHBSS containing 100 μM sulfobromophthalein to allow for the deesterification of the dye. Lung slices were mounted in the perfusion chamber as described above.

Fluorescence imaging was performed using a custom-built video-rate confocal microscope (Sanderson and Parker, 2003) modified to capture four channels simultaneously (Sanderson, 2004). In brief, the 488-nm line of an argon or diode laser was scanned across the specimen with two oscillating mirrors (for X- and Y-scan) in an inverted microscope. The resultant fluorescence (>510 nm) was detected by a photomultiplier tube (PMT). A video frame capture board (Raven; Bit Flow, Inc.) digitizes the PMT signal (1 PMT per channel) to form an image (480 pixels × 400 lines) that was recorded using the software Video Savant to a hard disk (a stripped SCSI volume). For time lapse, a recording rate of 2 Hz was used, otherwise video rate (30 Hz) was used. For the higher speed recordings of Ca2+ waves and elemental Ca2+ events, images were acquired at 60 Hz (480 pixels × 170 lines). A line-scan analysis of these images was performed by extracting a row of pixels from each image and placing them sequentially, as a time sequence, in a single image. Alternatively, changes in fluorescence intensity within the images were analyzed by selecting regions of interest (ROI) of ~5 × 5 pixels in a single SMC. Average fluorescence intensities of an ROI were obtained, frame-by-frame, using the Scion Image software with custom written macros that allow tracking of the ROI within an SMC as it moved with contraction. When necessary, a bleach correction was calculated from the bleaching rate observed during a period of 30 s before the perfusion of drugs. Final fluorescence values were expressed as a fluorescence ratio (F/F0) normalized to the initial fluorescence (F0).

Immunocytochemistry

Lung slices were washed in sHBSS, fixed with cold 100% acetone (−20°C) for 20 min, and washed in sHBSS containing 1 mg/ml BSA (sHBSS-BSA). Antibodies were diluted 1:250 in sHBSS-BSA. The slices were incubated for 1 h at room temperature with a FITC-conjugated mouse monoclonal antibody against α-smooth muscle actin (Sigma-Aldrich). Slices were washed three times in sHBSS-BSA, and fluorescence was recorded with the confocal microscope using 488 nm excitation light and a bandwidth >510 nm for emission. Phase-contrast images were recorded simultaneously with the fluorescence images by using a second PMT to detect the laser light transmitted by the specimen.

Statistics

A paired Student’s t test was used to test for significant differences between means. All statistical values are expressed as mean ± SEM.

Online Supplemental Material

Videos consisting of sequences of phase-contrast or fluorescence images were produced in Video Savant by exporting the images as “mpeg” files. To show the time of addition of agonists, labels were added to each frame using a custom written script file. Videos are presented in time lapse and the playback speed is indicated for each video. Videos 1–4 are available at http://www.jgp.org/cgi/content/full/jgp.200409216/DC1.

RESULTS

Characteristics and Morphology of Lung Slices

Throughout the lungs, the intrapulmonary airways and arteries have a close anatomical association that follows a parallel course. As a result, an airway and an accompanying arteriole (a bronchiole–arteriole pair) was easily identified and visualized in a single microscopic field of view (Fig. 1, A and B). This also makes the identification of pulmonary veins (not shown) easier because they are found as individual structures at some distance away from the bronchiole–arteriole pair. This anatomical separation precludes a direct comparison
The localization of SMCs and the contractile responses of an airway and arteriole in a lung slice induced by ACH, 5-HT, and KCl. (A) A lung slice was fixed and stained with FITC-conjugated antibodies against smooth muscle \( \alpha \)-actin. Fluorescence and phase-contrast images were recorded with a confocal microscope. The fluorescence image was assigned a green pseudo-color and superimposed on the phase-contrast image to show colocalization. The final image consists of a montage of six different images. The bronchiole airway (A) and the accompanying arteriole (a) are surrounded by alveolar parenchyma. A prominent layer of SMCs (green) is located below the epithelial (EPC) and endothelial (ENC) cells of the bronchiole and arteriole, respectively. (B) A series of phase-contrast images, recorded at different times (indicated by arrows in C) showing the appearance of an airway and arteriole before (1) and after stimulation with 1 \( \mu \)M ACH (2), 1 \( \mu \)M 5-HT (3), and 100 mM KCl (4). (C) The cross-sectional area of the lumen of an airway (blue line) and arteriole (red line) with respect to time in response to ACH, 5-HT, and KCl (top bars). ACH induced a contraction of the airway but not of the arteriole. 5-HT induced a greater contraction in the arteriole than in the airway. KCl induced twitching in both the airway and arteriole and a sustained contraction of the arteriole. Upon washout of ACH, the airway quickly relaxed, attaining 80–90% of its initial size within 3 min, but continued to slowly relax over the next 5–7 min. In contrast to the airways, the arterioles had no contractile response to ACH (Fig. 1 C, red line). The exposure of the same bronchiole–arteriole pair to 1 \( \mu \)M 5-HT induced the contraction of both the airway and arteriole (Fig. 1, B and C). In the airway, 5-HT induced a contraction at a similar rate compared with that induced by ACH and reduced the luminal area by...
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41 ± 5% after 5 min (n = 9 slices from 3 mice). In the arteriole, 5-HT induced an initial fast contraction that reduced the lumen by up to 80% within 2 min. This was followed by a second phase of slower contraction. The average arteriole luminal area was reduced by 75 ± 6% after 5 min (n = 6 slices from 3 mice). Upon 5-HT washout, both the airway and arteriole relaxed; however, it is important to note that the airway relaxed quickly, whereas the arteriole relaxation had a delayed onset and occurred more slowly (Fig. 1 C).

The same bronchiole–arteriole pair responded to isotonic sHBSS containing 100 mM KCl with a large reduction of the arterial lumen but only a small reduction of the airway lumen (Fig. 1, B and C). However, unlike the sustained contraction induced by ACH and 5-HT, the contraction induced by KCl was irregular or spasmodic (Video 1). This was especially evident in the airway where uncoordinated transient contractions (or twitching) of individual airway SMCs only resulted in a small decrease in luminal area. After 5 min, KCl had induced a luminal reduction in airways of 14 ± 3% (n = 7 slices from 3 mice) and arterioles of 64 ± 8% (n = 6 slices from 3 mice). Removal of the KCl resulted in arteriole and airway relaxation although some airways continued to display transient contractions (four out of seven).

These results indicate that the SMCs of airways and arterioles respond very differently to different agonists. Consequently, in this paper, we have focused on characterizing and correlating the contractile responses and the underlying Ca²⁺ signaling of the airway SMCs to 5-HT, ACH, and KCl. The responses of the arteriole SMCs to these agonists are addressed in a separate study (Perez and Sanderson, 2005).

Concentration Dependence of Airway Contraction to 5-HT, ACH, and KCl

To determine the relative sensitivity of the contraction of the airways to 5-HT, ACH, and KCl, we measured the changes in luminal area in response to sequentially increasing concentrations of agonist (Fig. 2). Increasing concentrations of 5-HT or ACH (from 0.01 to 0.5 µM) induced an increasing reduction in luminal size (Fig. 2, A and C). At low concentrations of 5-HT or ACH (0.01 µM), the airway contraction was small and consisted of uncoordinated twitching of the airway wall. At higher agonist concentrations, the airway contraction proceeded quickly and smoothly without twitching. While a maximal luminal reduction was induced by concentrations of ≥1 µM for each agonist, the maximal response to 5-HT was less than that induced by the maximal concentration of ACH (Fig. 2 C). This relative sensitivity was verified in each lung slice by the addition of the complementary agonist at the end of each experiment (Fig. 2 A). The addition of 10 µM ACH, after 10

Figure 2. The effect of concentration on 5-HT–, ACH–, and KCl-induced airway contraction. Lung slices with airways of similar sizes were stimulated with increasing concentrations of 5-HT, ACH, or KCl (perfusion time of 8 min). Relaxation of the airways between exposure to agonists or KCl was achieved by washing with sHBSS for 10 min. An image was recorded every 2 s and the airway lumen cross-sectional area was calculated and plotted with respect to time. (A) 5-HT induced a concentration-dependent increase in the contraction of airways in the range of 0.01–0.5 µM. Stimulation with 10 µM ACH induced a larger contraction as compared with 10 µM 5-HT. (B) A representative experiment showing the effect of increasing isotonic concentrations of KCl on the contraction of airways. (C and D) Summary of the concentration-dependent contractility of the airways to (C) ACH (open squares) and 5-HT (closed circles) or (D) KCl, calculated after 5 min of agonist or KCl exposure. Each point represents the mean ± SEM from at least three different experiments on different slices from at least two mice. For each agonist, the contractility data were fitted with a logistic function curve.

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μM 5-HT, resulted in a larger contraction (69% and 59%, respectively; Fig. 2 A). Conversely, the addition of 10 μM 5-HT, after the addition of 10 μM ACH, resulted in a smaller airway contraction (38% and 49%, respectively). 5-HT induced a smaller airway contraction than ACH at all concentrations (Fig. 2 C). An average maximal contraction of 48 ± 4% (n = 5 from 3 mice) and 59 ± 2% (n = 6 from 3 mice) was induced by 10 μM 5-HT and ACH, respectively. The half maximal concentration for airway contraction (EC50) was 31 and 30 nM for 5-HT and ACH, respectively.

Exposure of the airway to a range of isotonic KCl concentrations between 25 and 150 mM commonly induced a transient contraction that subsided into a smaller sustained contraction of the lumen that varied little over the range of KCl concentrations tested (Fig. 2, B and D). It is worth noting that, at all KCl concentrations, the small overall contraction was very similar to that induced by a very low concentration of agonist and was achieved by uncoordinated twitching. However, the maximum response to KCl was small, consisting of a contraction of only 11% ± 1% in response to 100 mM KCl (n = 4 from 3 mice). The order of the sensitivity of the contractile response of airways was ACH ≈ 5-HT >> KCl.

**Ca2+ Oscillations in Airway SMCs Induced by 5-HT and ACH**

In response to 1 μM 5-HT, the SMCs of the airway responded with an initial increase in [Ca2+]i, that was quickly followed by the occurrence of oscillations in [Ca2+]i (Fig. 3). These Ca2+ oscillations occurred asynchronously, with each cell displaying repetitive increases in [Ca2+]i, at different times with respect to neighboring SMCs (Fig. 3; Video 2, available at http://www.jgp.org/cgi/content/full/jgp.200409216/DC1). Individual Ca2+ oscillations occurred as Ca2+ waves propagating throughout the length of the cell (Video 3; also shown in Fig. 13), but no Ca2+ waves were observed to spread from one SMC to an adjacent SMC. The initiation of the Ca2+ oscillations in the SMCs was accompanied by a sustained contraction of the airway; the SMCs shortened and were displaced toward the airway lumen (Video 2).

With the exception of differences in frequency, the basic characteristics of the Ca2+ signals induced by ACH appeared similar to those induced by 5-HT (Fig. 4). In general, the pattern of Ca2+ oscillations consisted of two phases. The initial phase consisted of low amplitude and high frequency Ca2+ oscillations superimposed on an elevated [Ca2+]i, that lasted ~1 min (Fig. 4, A and C). During the second phase, the Ca2+ oscillations occurred with a stable frequency and similar amplitude superimposed on a decreasing baseline of [Ca2+]i, (Fig. 4, B and D, and Fig. 8). This second phase persisted for the remaining time while the agonist was present (see Fig. 8, C and D). Each individual Ca2+ oscillation consisted of an initial rapid Ca2+ transient followed by a slower phase of declining [Ca2+]i. The mean duration of the Ca2+ oscillation in 1 μM 5-HT or ACH was 0.7 ± 0.2 s or 0.8 ± 0.2 s, respectively. Although the magnitude of the increase of the fluorescence (F/F0) during the first and subsequent Ca2+ oscillations fluctuated between values of 1.5 and 3.0, no major differences between agonists were identified.

In the range from 10-8 to 10-6 M, both 5-HT and ACH induced a concentration-dependent increase in
the frequency of the Ca$^{2+}$ oscillations calculated during the second phase (Fig. 4 E). At all concentrations, 5-HT induced a Ca$^{2+}$ oscillation frequency that was slower than that induced by ACH. For example, 1 μM 5-HT induced a frequency of 18 ± 2 cycles min$^{-1}$ ($n$ = 5 slices of 3 animals) while 1 μM ACH induced a frequency of 26 ± 2 cycles min$^{-1}$ ($n$ = 6 slices of 3 animals, $P$ = 0.018, t test.). The half maximum concentration was 88 and 45 nM for 5-HT and ACH, respectively.

**Ca$^{2+}$ Signaling in Airway SMCs Induced by KCl**

High concentrations of KCl also induced increases in [Ca$^{2+}$]$_i$ and Ca$^{2+}$ oscillations (Fig. 5; Video 4). However, the KCl-induced Ca$^{2+}$ oscillations occurred more slowly and an initial phase of high frequency Ca$^{2+}$ oscillations was absent. The frequency of the Ca$^{2+}$ oscillations induced by KCl was low (one to three per minute) at all concentrations tested (25–100 mM; Fig. 5 D). An average frequency of 2.8 ± 1.4 cycles min$^{-1}$; ($n$ = 4 slices of 3 animals) occurred at 50 mM KCl but this was not significantly different from the frequencies recorded at higher or lower KCl concentrations. Each Ca$^{2+}$ transient or oscillation had a long duration (11.8 ± 2.6 s) (Fig. 5 B). In some cases, the Ca$^{2+}$ oscillations of neighboring cells had similar frequencies and appeared to be synchronized (four airways, Fig. 5). However, in other cases, neighboring cells showed asynchronous Ca$^{2+}$ oscillations with different frequencies (four airways). In each case, the Ca$^{2+}$ oscillations were accompanied by local transient contractions of either multiple or individual cells (Fig. 5 C; Video 4, available at http://www.jgp.org/cgi/content/full/jgp.200409216/DC1).

**Correlation of Airway Contraction with Ca$^{2+}$ Oscillation Frequency**

The correlation between the increasing frequency of the Ca$^{2+}$ oscillations and the increasing airway contraction with increasing concentrations of 5-HT and ACH suggests that frequency of the Ca$^{2+}$ oscillations determines the extent of airway contraction (Fig. 6). By contrast, an increasing correlation between airway contraction and the frequency of Ca$^{2+}$ oscillations induced by KCl did not exist because the frequencies of the Ca$^{2+}$ oscillations induced by a range of KCl concentrations were all very low and similar (Fig. 6). However, the contraction induced by these low frequency Ca$^{2+}$ oscillations in response to high KCl matched the contraction induced by low frequency Ca$^{2+}$ oscillations induced by low doses of agonists.
Type of 5-HT Receptor Involved in Airway Contraction

Because 5-HT2 and 5-HT3 receptors are known to produce increases in $\left[\text{Ca}^{2+}\right]_i$, 5-HT-specific antagonists and agonists were used to determine which receptor type mediates airway contraction. Ketanserin, a 5-HT2-specific antagonist, at 10 nM blocked the contraction induced by 1 μM 5-HT (Fig. 7 A). In addition, sequential stimulation with 10⁻⁸, 10⁻⁷, and 10⁻⁶ M DOI (2, 5-dimethoxy-4-iodoamphetamine hydrochloride), a 5-HT2-specific agonist, induced reversible airway contraction (three airways from three mice). On the other hand, SR 57227 (4-amino-1-(6-chloro-2-pyridyl)-piperidine hydrochloride), a 5-HT3 agonist, did not induce a substantial contraction at 10⁻⁷ or 10⁻⁶ M (the decrease in airway lumen area was 10%, three airways from three mice). These results suggest that the 5-HT2 receptor is responsible for mediating 5-HT responses.

Alternative Mechanisms of Action for 5-HT and KCl

Because a cholinergic pathway has been proposed as a mechanism by which 5-HT induced airway contraction in trachea and isolated lungs (Levitt and Mitzner, 1989; Eum et al., 1999; Fernandez et al., 1999; Held et al., 1999; Moffatt et al., 2004), we examined the effect of atropine on the 5-HT responses of lung slices to determine if any 5-HT effects occurred indirectly. Atropine (1 μM) had no effect on the airway contractile response (Fig. 7 B) when added, either before or after exposure to 1 μM 5-HT. By contrast, 1 μM atropine totally abolished the airway contractile response induced by 1 μM ACH. Similarly, 1 μM atropine induced a full relax-
traces of at least three different slices from three mice. Blocked or relaxed ACH-induced contraction. Representative no effect on 5-HT–induced airway contraction but completely presence of 1

5-HT2 antagonist) as indicated by bars. (B) An airway sequentially addition, it should be noted that during agonist or KCl

Figu...
both nifedipine and Ni
2
+ completely blocked airway contraction stimulated by high KCl. These results indicate that while L-type Ca
2
+ channels may have a significant role in KCl-induced contraction, they have little influence in agonist-induced contraction and suggest that other Ca
2
+ channels serve to sustain contraction.

To investigate the cause of the slow relaxation of the airway observed during the stimulation with ACH in the continued presence of Ni
2
+, we simultaneously measured the changes in SMC [Ca
2
+]i and contraction of the airway (Fig. 9 C). After the initial increase in [Ca
2
+]i and the onset of Ca
2
+ oscillations, the frequency of Ca
2
+ oscillations progressively decreased and this was accompanied by the relaxation of the airway. After removal of Ni
2
+, but still in the presence of ACH, the frequency of the Ca
2
+ oscillations increased again and the airway recontracted. These results suggest that the Ni
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+ blocked Ca
2
+ channels that contribute to the maintenance of the Ca
2
+ oscillations and perhaps serve to refill internal Ca
2
+ stores.

Figure 8. Role of extracellular Ca
2
+ during the contraction and the Ca
2
+ signaling of airway SMCs induced by 5-HT and ACH. (A and B) Contractile responses of airways in different lung slices sequentially exposed (top bars) to 1 μM 5-HT and 1 μM ACH in the presence or absence of extracellular Ca
2
+. Representative traces of five airways from three mice. (C-H) The changes in [Ca
2
+]i in small ROIs of single SMCs were determined in lung slices stimulated with (C, E, and G) 1 μM 5-HT or (D, F, and H) 1 μM ACH in the presence or absence of extracellular Ca
2
+. Although Ca
2
+ oscillations persisted in the presence of extracellular Ca
2
+, they progressively reduce their frequency and stopped in the absence of extracellular Ca
2
+. (G and H) The frequency of agonist-induced Ca
2
+ oscillations, calculated every 20 s, in the presence (squares) or absence of Ca
2
+ (triangles) with respect to time. Symbols and bars are the mean ± SEM of five to eight SMCs in five paired experiments from different lung slices from three mice.
The Contribution of Ca\textsuperscript{2+} Influx to Airway Contraction

To further investigate the mechanisms and Ca\textsuperscript{2+} source used during agonist and KCl-induced contractions and Ca\textsuperscript{2+} oscillations, lung slices were exposed to caffeine to empty internal stores by opening ryanodine receptors (RyRs). In response to sustained exposure of caffeine, the airways displayed a single transient contraction (Fig. 10 A). The maximal contraction (38 ± 6%, \(n = 12\) slices from 4 mice) was attained within 8–10 s, and after 1 min, the airways were almost completely relaxed (5 ± 3%, \(n = 12\) slices from 4 mice).

Under similar conditions, caffeine induced an initial transient increase in [Ca\textsuperscript{2+}], that was followed by a sustained plateau of increased [Ca\textsuperscript{2+}], (Fig. 10 B). The average peak and plateau ratio values were 2.8 ± 0.4 and 1.7 ± 0.3, respectively (\(n = 6\) slices from 2 animals). Simultaneous recording of the changes in airway lumen area showed that the transient contraction accompanied the transient increase in Ca\textsuperscript{2+}. However, immediately following the Ca\textsuperscript{2+} transient, the airway relaxed and remained relaxed during the decline in Ca\textsuperscript{2+} and during the extended period of elevated Ca\textsuperscript{2+}. The caffeine-induced plateau of elevated Ca\textsuperscript{2+} was quickly abolished when extracellular Ni\textsuperscript{2+} was withdrawn (Fig. 10 C). Similarly, the elevated Ca\textsuperscript{2+} plateau was reduced when extracellular Ni\textsuperscript{2+} was added (Fig. 10 D). These manipulations did not alter the relaxed state of the airway. In the absence of extracellular Ca\textsuperscript{2+}, caffeine was still capable of inducing a transient Ca\textsuperscript{2+} increase and contraction, but a sustained increase in Ca\textsuperscript{2+} did not occur (Fig. 10 E). However, the replacement of extracellular Ca\textsuperscript{2+} resulted in a rapid and sustained increase in Ca\textsuperscript{2+} that was accompanied by a very small contraction of the airway (3.3 ± 1.9\%, \(n = 5\) slices from 2 mice). These results indicate that caffeine initiated the emptying of the internal Ca\textsuperscript{2+} stores and that this induced an influx of extracellular Ca\textsuperscript{2+}. Interestingly, the transient of Ca\textsuperscript{2+} only induced a transient airway contraction while the sustained Ca\textsuperscript{2+} influx did not maintain sustained contraction.

Role of Intracellular Ca\textsuperscript{2+} Stores During Stimulation with 5-HT, ACH, and KCl

With this basic information on how caffeine acts on lung slices, we examined the effect of caffeine on agonist- and KCl-induced contraction. Consistent with the previous experimental results, caffeine completely, but reversibly, blocked airway contraction and the generation of Ca\textsuperscript{2+} oscillations (\(n = 3\) slices from 2 mice) induced by 5-HT and ACH (Fig. 11, A and B). These re-
results further suggest that contraction and Ca\(^{2+}\) oscillations are dependent on internal Ca\(^{2+}\) stores.

To further test if refilling of Ca\(^{2+}\) stores depends on a Ni\(^{2+}\)-sensitive Ca\(^{2+}\) influx, we examined the effect of caffeine and Ni\(^{2+}\) on the response to ACH (Fig. 11 C). In the presence of Ni\(^{2+}\), the contractile response of an airway to successive exposures of caffeine progressively declined (Fig. 11 C). This indicated that the internal Ca\(^{2+}\) stores were progressively depleted. The subsequent stimulation with ACH only induced a small contraction (19.6 ± 4.6% relative to the control contraction) that relaxed (to 6.5 ± 0.6%) after 8 min (3 slices from 2 mice). Upon removal of Ni\(^{2+}\), ACH once again induced a strong contraction (86.9 ± 6.0% and 99.6 ± 10% after 8 min). In control experiments, in the absence of Ni\(^{2+}\), airway contraction did not decline in response to a similar series of caffeine exposures and this treatment did not affect the subsequent ACH-induced contraction (2 airways from 2 mice). These results indicate the presence of a Ni\(^{2+}\)-sensitive Ca\(^{2+}\) influx to refill Ca\(^{2+}\) stores to sustain contraction.

Surprisingly, caffeine also completely blocked airway contraction or twitching induced by KCl (Fig. 12 A). The twitching response to KCl was also blocked with cyclopiazonic acid (CPA), an inhibitor of SR Ca\(^{2+}\) pump (SERCA) (Fig. 12 B). These results suggest that KCl-induced twitching as well as 5-HT– and ACH-induced contractions were dependent on cycles of release and reloading of the Ca\(^{2+}\) stores.

**Ca\(^{2+}\) Waves and Elemental Ca\(^{2+}\) Events in Airway SMCs**

To explore the mechanisms mediating fast Ca\(^{2+}\) oscillations induced by agonists and slow Ca\(^{2+}\) oscillations induced by KCl from internal stores, we compared the
spatio-temporal properties of the Ca\(^{2+}\) oscillations induced by 5-HT, ACH, and KCl in airway SMCs using high speed recordings of the changes in \([\text{Ca}^{2+}]_i\). Line-scan plots from the longitudinal axis of single SMCs showed that Ca\(^{2+}\) oscillations induced by 5-HT and ACH consisted of Ca\(^{2+}\) waves that propagated along the entire longitudinal axis of the cell (Fig. 13). The average velocity of the Ca\(^{2+}\) waves induced by 5-HT and ACH was 27 ± 2 μm/s and 43 ± 4 μm/s, respectively. In general, as indicated by the consistent pattern of the line-scan analysis, the Ca\(^{2+}\) waves appeared to be initiated at specific locations within the cell. However, successive waves could change their initiation site and propagate in the opposite direction (Fig. 13).

In contrast to the regular patterns of agonist-induced Ca\(^{2+}\) waves, KCl-induced waves were preceded by multiple small, localized, and transient Ca\(^{2+}\) increases (or “elemental Ca\(^{2+}\) events”) that did not propagate through the whole cell (Fig. 13, arrows). These elemental Ca\(^{2+}\) events were also initiated from specific locations within a cell at an average frequency of 7 ± 4 per min (8 cells from 5 slices from 2 mice). However, their frequency increased with time, and was fastest just before a Ca\(^{2+}\) wave was initiated. The Ca\(^{2+}\) waves induced by KCl had slower propagating velocities (18 ± 2 μm/s) and a longer duration than those induced by 5-HT or ACH (Figs. 4, 5, and 13). After each Ca\(^{2+}\) wave had subsided, a refractory period elapsed before the elemental Ca\(^{2+}\) events started to occur again. KCl-induced Ca\(^{2+}\) waves were usually initiated from one location.
that showed the highest frequency of elemental signaling. Each elemental Ca\textsuperscript{2+} event occurred with a diameter of 4 $\pm$ 2 $\mu$m, had a rise-to-peak time of 80 $\pm$ 30 ms and a first order exponential decay of 420 $\pm$ 115 ms (Fig. 13, inset, $n$ = 4 cells from 4 slices from 2 mice). Before the addition of KCl, the elemental Ca\textsuperscript{2+} events were not observed; after the removal of KCl, the elemental Ca\textsuperscript{2+} events were virtually abolished (Fig. 13, HBSS line). Ca\textsuperscript{2+} waves and elemental Ca\textsuperscript{2+} events were not observed during KCl stimulation in the presence of caffeine or CPA.

**DISCUSSION**

We further developed the lung slice preparation to compare the contractile responses of intrapulmonary airways and arterioles and the underlying changes in [Ca\textsuperscript{2+}]\textsubscript{i} in the associated SMCs. Lung slices are well suited for these studies because the airways and blood vessels can be easily identified at regions which are considered important in the development of asthma and pulmonary hypertension and a link between the responses of the SMCs and the physiology of airways and blood vessels can be made. In addition, the robust responses of the airways and arterioles allow a quantitative evaluation of the contractile response to different agonists or concentrations of the same agonist. Other important qualities of lung slices include the ability to simultaneously study the response of two types of SMCs to the same conditions, a viability of several days and the use of serial sections and transgenic animals (Kotlikoff et al., 2004).

Airway or arteriole contraction is limited by the balance between the contractile forces produced by the SMCs and the recoil forces generated by surrounding alveolar tissue. In the animal, the recoil forces are established by lung inflation with air (Ding et al., 1987). In lung slices, the recoil forces are established by inflation with agarose. Because overinflation will reduce the contractile response, it is important to adjust the inflation volume of agarose to be about equal (but not greater than) the volume of the chest cavity (1–1.4 ml). At this volume, a reduction in the airway area of 50–60% in response to a maximal dose of 5-HT or ACH was consistently observed. While differences in contractile responses could be attributed to differences in the inflation volume, this possibility is minimized by using the same lung slice to evaluate multiple drugs. In addition, the similarity of the responses of slices to agonists from different mice suggests that the lung inflation was similar between each preparation.

The airways and arterioles of mouse lung slices responded differently to agonists and KCl. In response to 5-HT, the airways and arterioles displayed a sustained contraction but in response to ACH only the airways displayed contraction. This contrasts with arterial perfusion of isolated lungs where 5-HT only induced a small increase in airway resistance and pulmonary artery pressure (Held et al., 1999); this difference might be explained by the fact that 5-HT was not in contact with the SMCs in perfused lungs. In response to KCl, the airways of lung slices displayed SMC twitching while the arterioles displayed contraction and twitching. To understand the mechanisms underlying these responses, it was necessary to characterize the Ca\textsuperscript{2+} responses of the SMCs. However, for clarity, we only report here the contractility and Ca\textsuperscript{2+} signaling of airway SMCs induced by agonists and KCl.
of arteriole SMCs are the subject of a second study where we develop a hypothesis to propose how different frequencies of Ca\(^{2+}\) oscillations regulate contraction of airway and arteriole SMCs (Perez and Sanderson, 2005).

We have found that 5-HT and ACH induced airway contraction in a similar manner although the contraction induced by ACH was greater than that induced by 5-HT. A greater contractile response to MCH, as compared with 5-HT, was also observed in isolated mouse lungs (Held et al., 1999) and trachea rings (Moffatt et al., 2004). However, 5-HT was also believed to act via a cholinergic pathway. In our lung slices, atropine had no effect on 5-HT–induced contraction of airways even though atropine completely blocked airway contraction induced by ACH. These results suggest that 5-HT does not act via a cholinergic pathway and that 5-HT and ACH may be putative regulators of the caliber of the small airways of the mouse.

Agonist-induced (5-HT or ACH) contraction of the airways was generally sustained, whereas the KCl-induced contraction was characterized by SMC twitching. This difference indicates that agonist-induced contraction does not require membrane depolarization (Bolton et al., 1999a), a hypothesis supported by the fact that 5-HT and ACH can act via G protein–coupled receptors. 5-HT and ACH, but not KCl, also initiated contraction in the absence of extracellular Ca\(^{2+}\) or in the presence of nifedipine, an antagonist of L-type Ca\(^{2+}\) channels. A similar finding that nifedipine only slightly relaxed tracheal SMCs contracted with ACH was reported by Kuo et al., (2003). However, both 5-HT and ACH only induced a transient contraction in the absence of extracellular Ca\(^{2+}\) or in the presence of Ni\(^{2+}\); results that indicate that external Ca\(^{2+}\) entry is necessary to maintain the sustained contraction. The persistent contraction of the airway in the presence of nifedipine suggests that L-type Ca\(^{2+}\) channels are not the route of Ca\(^{2+}\) entry. However, voltage-sensitive T-type Ca\(^{2+}\) currents have been found in bronchial SMCs and may play a role in excitation–contraction and the refilling of Ca\(^{2+}\) stores (Janssen, 1997; Yamakage et al., 2001). T-type Ca\(^{2+}\) channels have little sensitivity to nifedipine but are blocked by Ni\(^{2+}\); consequently, we cannot rule out the possibility that T-type Ca\(^{2+}\) channels are involved in refilling the Ca\(^{2+}\) stores. Because of the less-specific effects of Ni\(^{2+}\), it is also likely that other membrane channels, such as store-operated or noncapacitative, receptor-operated Ca\(^{2+}\) channels (transient receptor potential channels), contribute Ca\(^{2+}\) entry in airway SMCs (Berridge et al., 2003; Li et al., 2003; Ay et al., 2004). The findings that L-type Ca\(^{2+}\) channel blockers are ineffective as therapeutic agents for asthma and that agonist-induced changes in membrane potential are inadequate to activate L-type Ca\(^{2+}\) channels in airway SMCs further de-emphasize the role suggested for L-type voltage-gated Ca\(^{2+}\) channels in airway contraction (Janssen, 2002).

Previous investigations of Ca\(^{2+}\) signaling in rat (Toloczko et al., 1995, 1997) or dog (Yang et al., 1997; Yang, 1998) tracheal SMCs used low-speed sampling systems to report that 5-HT induced an initial transient followed by a sustained elevation in [Ca\(^{2+}\)]. By contrast, we found with video-rate confocal microscopy that 5-HT induces repetitive transients in [Ca\(^{2+}\)], or Ca\(^{2+}\) oscillations in airway SMCs. In most respects, the 5-HT–induced Ca\(^{2+}\) oscillations were similar to those induced by ACH, both in this and previous studies with lung slices (Bergner and Sanderson, 2002a, 2003) or isolated tracheal airway preparations (Prakash et al., 1997, 2000; Roux et al., 1997; Kuo et al., 2003). The Ca\(^{2+}\) oscillations persisted with a steady frequency and usually originated at one end of the cell and spread toward the other end as a Ca\(^{2+}\) wave, although the direction of the Ca\(^{2+}\) waves could be reversed. The Ca\(^{2+}\) waves were unsynchronized between neighboring cells and did not propagate to adjacent cells, suggesting that each wave originated within each cell. It is important to note that each Ca\(^{2+}\) oscillation did not generate a twitch of contraction but that the SMCs maintained a steady contractile state.

A significant characteristic of agonist-induced Ca\(^{2+}\) oscillations was the fact that the frequency of the Ca\(^{2+}\) oscillations increased with the concentrations of 5-HT or ACH. Similarly, the extent of the airway contraction was concentration dependent over the same range. This relationship between contraction and Ca\(^{2+}\) oscillation frequency suggests that the size of the airway contraction is regulated by frequency modulation of the changes in [Ca\(^{2+}\)], (Berridge et al., 2003). Consistent with this idea is the fact that we could not establish a relationship between airway contraction and the magnitude or duration of the Ca\(^{2+}\) oscillations. Agonist-induced Ca\(^{2+}\) oscillations could initially have large amplitudes, and although this amplitude could decline with time, it did not alter the level of contraction. Similarly, slowing the Ca\(^{2+}\) oscillation frequency (e.g., with 0 extracellular Ca\(^{2+}\) or Ni\(^{2+}\)) induced relaxation but did not alter the oscillation amplitude. Slow KCl-induced Ca\(^{2+}\) oscillations had a similar magnitude but a much longer duration (≥10 times) than the oscillations induced by agonists, yet the contraction induced by KCl was smaller. A similar relationship between the Ca\(^{2+}\) oscillatory frequency and contractility was reported for ACH (Bergner and Sanderson, 2002a) and ATP (Bergner and Sanderson, 2002b) in lung slices and for ACH in SMCs bundles isolated from porcine trachea (Kuo et al., 2003).

The most likely mechanism for the generation of 5-HT– or ACH-induced Ca\(^{2+}\) oscillations is agonist acti-
vation of PLC via a G protein–coupled receptor to generate inositol 1,4,5-trisphosphate (IP3) that, in turn, initiates repetitive cycles of Ca2+ release and uptake from the SR via the IP3 receptor (IP3R) (Roux et al., 1997; Pabelick et al., 2001; Bergner and Sanderson, 2002a; Janssen, 2002). However, caffeine inhibits the contrac-tile response to ACH and 5-HT, a result suggesting that internal stores with RyRs contribute to the Ca2+ fluxes occurring during Ca2+ oscillations. While previous studies indicate that ACH binds to an M3 muscarinic receptor, 5-HT appears to act via the 5-HT2, Gq protein–coupled, receptor (Hoyer et al., 2002) because airway contraction was inhibited by ketanserin, a 5-HT2 receptor blocker, and stimulated by DOI, a 5-HT2 receptor agonist. Although 5-HT3 receptors can elevate [Ca2+]i, via a Ca2+ influx (Reeves and Lummis, 2002), its role appears minimal because 5-HT–induced increases in [Ca2+]i in the absence of extracellular Ca2+ and the selective 5-HT3 receptor agonist SR-57227 did not stimulate the airway contraction.

The IP3Rs, RyRs, and SERCA all appear to participate in the generation of Ca2+ oscillations, but the mechanisms that regulate the Ca2+ oscillation frequency are unclear. One idea is that the Ca2+ oscillation rate is influenced by the basal [Ca2+]i, which increases the sensitivity of the RyR to CICR and SERCA pump activity (Prakash et al., 2000; Pabelick et al., 2001). An alternative idea is that the frequency is regulated by the intracellular concentration of IP3 acting on the IP3R (Berridge et al., 2003) and that the elevated [Ca2+]i is a consequence of increases in Ca2+ oscillation frequency. Because CICR via the IP3R may also be influenced by [Ca2+]i, it may take a short period to establish a steady state for the Ca2+ oscillations after an initial surge of IP3 associated with agonist stimulation. We have observed an initial increase in the basal [Ca2+]i, with high frequency Ca2+ oscillation, but it is interesting to note that the airway contraction remains steady, if not increases, during this stabilization period. Differences in the frequency rate between cells could be the result of heterogeneities in internal and external receptor expression.

Before exploring the hypothesis that KCl-induced contraction is mediated by the depolarization of SMCs, we ruled out the alternative hypothesis that KCl acts by stimulating the local release of neurotransmitters for several reasons. First, KCl stimulated SMC twitching in the presence of ketanserin or atropine, 5-HT and ACH receptor antagonists, and apyrase, an ATPase. Second, the airways did not respond to phenylephrine, an α1-agonist. These results indicate that a release of ACH, 5-HT, ATP, or noradrenaline from nerve terminals does not explain the contraction stimulated by KCl. Third, because our experiments were performed with constant perfusion, any neurotransmitter released would be quickly washed away and could not generate a sustained response. And, finally, the Ca2+ responses and contraction of the SMCs to KCl were very different to those induced by agonists.

The hypothesis for the mechanism by which KCl triggers Ca2+ oscillations is also constructed from several lines of evidence. First, while the KCl-induced Ca2+ oscillations were characterized as long-lasting Ca2+ waves that occurred at low frequencies, these Ca2+ waves were preceded by multiple, transient Ca2+ increases or “elemental Ca2+ events” that were similar to Ca2+ sparks or Ca2+ puffs observed in other cells. Second, these KCl-induced Ca2+ oscillation and events were abolished by caffeine and CPA, which suggests that these elemental Ca2+ signals involve RyRs and the release of intracellular Ca2+. The prolonged period of the KCl-induced Ca2+ wave suggests an extensive emptying of the internal Ca2+ store, and this is consistent with the development of a refractory period immediately following the Ca2+ waves in which no Ca2+ events were observed. Third, the propagation velocity of the KCl-induced Ca2+ waves is slow and similar to that induced by agonists, which supports the idea that the transient Ca2+ oscillation results from CICR from internal stores rather than from a Ca2+ influx initiated by fast propagating changes in membrane potential. Fourth, in the presence of extracellular KCl, oscillations in membrane potential would not be expected, but if they did occur, they would be expected to propagate to adjacent SMCs; however, Ca2+ waves induced by KCl occurred asynchronously between adjacent cells. And, finally, the KCl-induced Ca2+ oscillations required extracellular Ca2+ influx and were sensitive to nifedipine and Ni2+.

From this data we hypothesize that the KCl-induced Ca2+ oscillations are the result of the following events. Initially, KCl induces membrane depolarization and initiates an influx of Ca2+ via L-type and/or T-type Ca2+ channels. The cell compensates for this rise in [Ca2+]i, by transporting the extra Ca2+ into the SR via SERCA pumps. Because of their limited capacity, the stores quickly overload as indicated by the increasing frequency of the elemental Ca2+ events, which reflect sensitized RyRs. Upon reaching a critical Ca2+ load, the elemental Ca2+ events trigger an extended phase of CICR via sensitized RyR to empty the store and generate a Ca2+ wave with a transient SMC contraction before initiating the cycle again.

It is unknown if the elemental Ca2+ events arise from pure clusters of RyRs or mixtures of RyR and IP3Rs. Ca2+ sparks, observed in other SMCs, occurred as localized and transient Ca2+ increases and had rise times of 20 ms and initial decay constants of 50 ms (Wellman and Nelson, 2003). The elemental Ca2+ events recorded here had longer rise times and decay constants. However, under conditions of Ca2+ overload, a Ca2+ spark can activate CICR of a neighboring cluster of
RyRs to form a compound Ca\(^{2+}\) spark (Wang et al., 2004). In cardiomyocytes, compound Ca\(^{2+}\) sparks can evolve into propagating Ca\(^{2+}\) waves (Cheng et al., 1993, 1996), and we consistently observed that KCl-induced Ca\(^{2+}\) waves were initiated from sites where localized Ca\(^{2+}\) events were occurring.

The KCl-induced Ca\(^{2+}\) waves occur at low frequencies and result in twitching in the airway SMCs; yet, under these conditions, there appears to be a steady influx of Ca\(^{2+}\) but this is incapable of maintaining a sustained contraction. A steady influx of Ca\(^{2+}\) can also be invoked by emptying the internal Ca\(^{2+}\) stores with caffeine, but the SMCs remain relaxed. This is consistent with the idea that the contraction of airway SMCs is mediated by the frequency of the Ca\(^{2+}\) oscillations instead of a sustained elevation of Ca\(^{2+}\). An alternative explanation for the lack of contraction is that caffeine acts as a phosphodiesterase inhibitor to reduce the Ca\(^{2+}\) sensitivity of the SMC (Hall, 2004). However, the inhibition of the KCl-induced Ca\(^{2+}\) oscillations and twitching by caffeine and CPA suggests a direct action of caffeine on the Ca\(^{2+}\) stores.

The fact that a steady Ca\(^{2+}\) influx, initially driven by a change in membrane potential, has little effect on the regulation of airway SMC contraction suggests that Ca\(^{2+}\) sparks (Bolton et al., 1999b; Zhuge et al., 2004) do not serve as a relaxation mechanism for bronchiolar SMCs. These Ca\(^{2+}\) events may counteract the overloading of the internal stores by membrane hyperpolarization when coupled to Ca\(^{2+}\)-activated K\(^{+}\) channels (BK channels) but it appears that this process only occurs in airways exposed to KCl. As a result, cyclic Ca\(^{2+}\) release from internal stores seems to be the most important signal to sustain airway SMC contraction, while Ca\(^{2+}\) influx through nifedipine-resistant, Ni\(^{2+}\)-sensitive channels is necessary to replenish the Ca\(^{2+}\) stores and maintain the frequency of the Ca\(^{2+}\) oscillations.

The sources of 5-HT in the lungs include pulmonary neuroendocrine cells that form neuroepithelial bodies in the airways of animals, including humans (Lauweryns et al., 1973; Junod, 1975; Gonmori et al., 1986; Prasada Rao and Mehendale, 1987; Ben-Harari et al., 1990) and mast cells (Wasserman, 1994). Although substantial amounts of 5-HT are also synthesized in the gut and stored in blood platelets, circulating 5-HT had a minor effect on airways (Held et al., 1999). Consequently, 5-HT that is released to the basolateral space in response to hypoxia or neural activity (Cutz et al., 1993; Lommel, 2001; Adriaensen et al., 2003) or by mast cells during de-granulation in an allergic response may serve as a paracrine stimulant of SMCs. The possibility that 5-HT serves as a putative regulator of SMCs is supported by the ability of SMCs to transport and metabolize 5-HT; actions that would inactivate 5-HT (Dodson et al., 2004).

Another interesting aspect of lung slices that requires further investigation is the influence, if any, of the airway epithelial cells on the contractile responses of the airway SMCs. While epithelial cells can release mediators such as prostaglandin E\(_2\) and nitric oxide that may relax SMCs (Folkeerts and Nijkamp, 1998), we did not observe any major changes in [Ca\(^{2+}\)] in the epithelial cells in response to ACH, 5-HT, or KCl that might stimulate this release. In addition, in our previous studies with lung slices, we found little or no effect of ATP on the release of relaxing factors from epithelial cells (Bergner and Sanderson, 2002b).

In conclusion, intrapulmonary airways respond to 5-HT and ACH with a contraction that is maintained by high frequency Ca\(^{2+}\) oscillations within the SMCs that arise from repetitive cycles of Ca\(^{2+}\) release and uptake by the SR and require extracellular Ca\(^{2+}\) for store refilling. By contrast, KCl-induced twitching of SMCs results from low frequency Ca\(^{2+}\) oscillations produced by an overfilling and uncontrolled release of internal Ca\(^{2+}\). Most importantly, the magnitude of the contraction of airway SMCs is regulated by the frequency of the Ca\(^{2+}\) oscillations.

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