The Contraction of Smooth Muscle Cells of Intrapulmonary Arterioles Is Determined by the Frequency of Ca^{2+} Oscillations Induced by 5-HT and KCl

Jose F. Perez and Michael J. Sanderson

Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655

ABSTRACT Increased resistance of the small blood vessels within the lungs is associated with pulmonary hypertension and results from a decrease in size induced by the contraction of their smooth muscle cells (SMCs). To study the mechanisms that regulate the contraction of intrapulmonary arteriole SMCs, the contractile and Ca^{2+} responses of the arteriole SMCs to 5-hydroxytrypamine (5-HT) and KCl were observed with phase-contrast and scanning confocal microscopy in thin lung slices cut from mouse lungs stiffened with agarose and gelatin. 5-HT induced a concentration-dependent contraction of the arterioles. Increasing concentrations of extracellular KCl induced transient contractions in the SMCs and a reduction in the arteriole luminal size. 5-HT induced oscillations in $[Ca^{2+}]_i$ within the SMCs, and the frequency of these Ca^{2+} oscillations was dependent on the agonist concentration and correlated with the extent of sustained arteriole contraction. By contrast, KCl induced Ca^{2+} oscillations that occurred with low frequencies and were preceded by small, localized transient Ca^{2+} events. The 5-HT–induced Ca^{2+} oscillations and contractions occurred in the absence of extracellular Ca^{2+} and were resistant to Ni^{2+} and nifedipine but were abolished by caffeine. KCl-induced Ca^{2+} oscillations and contractions were abolished by the absence of extracellular Ca^{2+} and the presence of Ni^{2+}, nifedipine, and caffeine. Arteriole contraction was induced or abolished by a $5\text{-}HT_2$–specific agonist or antagonist, respectively. These results indicate that 5-HT, acting via $5\text{-}HT_2$ receptors, induces arteriole contraction by initiating Ca^{2+} oscillations and that KCl induces contraction via Ca^{2+} transients resulting from the overfilling of internal Ca^{2+} stores. We hypothesize that the magnitude of the sustained intrapulmonary SMC contraction is determined by the frequency of Ca^{2+} oscillations and also by the relaxation rate of the SMC.

KEY WORDS: confocal microscopy • pulmonary hypertension • airways • arteriole • serotonin

INTRODUCTION

Pulmonary hypertension (PH) is characterized by a progressive elevation of pulmonary arterial pressure and pulmonary vascular resistance, which results in heart failure. The increase in pulmonary resistance results from vasconstriction and/or structural changes, including smooth muscle cell (SMC) hypertrophy and hyperplasia. The etiology of primary PH is poorly understood, whereas secondary PH usually results from hypoxia associated with respiratory disease or high altitude breathing (Farber and Loscalzo, 2004). The pulmonary artery distributes blood to the alveoli via intrapulmonary arteries and arterioles that follow the branching pattern of the airways. However, along the pulmonary artery, the phenotype and physiology of the SMCs can vary, and in different regions, SMCs may respond differently to vasoactive factors (Frid et al., 1997). Blood returns to the heart via larger veins that follow a route distinct from that of arterioles and airways. Consequently, an understanding of the physiological factors that regulate the resistance of the pulmonary circulation is necessary to identify the pathological basis of hypertension.

The contractile mechanisms of the pulmonary arteries have been generally investigated in SMCs isolated from large extrapulmonary arteries (Barnes and Liu, 1995). While these studies provided valuable information, it is difficult to know how well the responses of SMCs of the pulmonary artery reflect the physiology of small intrapulmonary arterioles. Therefore, we examined the responses of small intrapulmonary arterioles in lung slices to correlate the Ca^{2+} signaling of the SMCs with the contraction. These arterioles were studied by modifying our lung slice preparation used with confocal microscopy (Bergner and Sanderson, 2002a,b, 2003), by temporarily filling the arterioles with gelatin. While we simultaneously examined the contractile responses of both the bronchioles and arterioles, we report here the responses of the

Correspondence to Michael J. Sanderson: Michael.Sanderson@umassmed.edu

The online version of this article contains supplemental material.

Abbreviations used in this paper: FM, frequency modulation; 5-HT, 5-hydroxytrypamine; IP_3, inositol 1,4,5-trisphosphate; PE, phenylephrine; PH, pulmonary hypertension; ROI, region of interest; SMC, smooth muscle cell.

arterioles. An accompanying paper reports the airway responses (Perez and Sanderson, 2005).

5-HT is a potent vasoconstrictor of pulmonary blood vessels in animals and humans (MacLean et al., 2000) and is thought to contribute to the development of primary PH and secondary PH mediated by hypoxia. Secondary PH may also be induced by amphetamine-derived appetite suppressants that act as 5-HT agonists (Eddahibi et al., 2002). Consequently, we investigated the contractile and $[Ca^{2+}]_i$ responses of arteriole SMCs to 5-HT. In addition, because membrane potential is important in contractility of other blood vessels, we examined the responses of intrapulmonary arterioles to KCl. We found that both 5-HT and KCl induced Ca^{2+} oscillations in the SMCs and that these Ca^{2+} oscillations were responsible for sustaining contraction. The frequencies of these Ca^{2+} oscillations were slower than those found in airway SMCs yet the contractile responses were larger. To resolve these data, we suggest that the contraction of the SMC is determined by the frequency of the Ca^{2+} oscillations as well as by the relaxation rate of the SMC; excessive contraction may therefore arise from a change in the Ca^{2+} signaling or relaxation rate of the SMCs.

MATERIALS AND METHODS

All the materials and methods used have been previously described in detail (Perez and Sanderson, 2005); only a brief outline is given here. Reagents were obtained either from Invitrogen and GIBCO BRL or Sigma-Aldrich. Hanks' balanced salt solution was supplemented with 20 mM HEPES buffer (sHBSS). A KCl salt solution (K^+-sHBSS) was prepared by substituting the sodium salts with potassium counterparts. High KCl isotonic solutions were prepared by mixing K^+-sHBSS with normal sHBSS. Hanks' "0" Ca^{2+} solution was prepared by supplementing sHBSS without Ca^{2+} and Mg^{2+} with 0.9 mM $MgSO_4$ and 1 mM Na_2H_2-EGTA.

Lung Slices

Lung slices were prepared from BALB/C inbred mice between 7 and 9 wk old. The trachea was cannulated with an intravenous catheter, and after opening the chest cavity, the collapsed lungs were reinflated with ~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. The lungs were deflated and refilled with a warm solution of 2% agarose (low gelling temperature) in sHBSS. Subsequently, a small volume of air was injected to flush the agarose-sHBSS out of the airways and into the distal alveolar space. The warm agar and the gelatin were gelled with cold sHBSS. A single lung lobe was removed and cut in serial sections of ~130 μm thickness with a vibratome at ~4°C starting at the lung periphery. Serum was not used because it contains 5-HT and growth factors (Abdullah et al., 1994). Slices were maintained in growth 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

Only arterioles which had not collapsed were used. Lung slices were mounted in a custom-made perfusion chamber and held in place with a small sheet of nylon mesh. A second coverglass edged with silicone grease was placed over the lung slice. Perfusion of the lung slice was performed by a gravity-fed perfusion system. The volume of the chamber was ~100 μl with a perfusion rate of 800 μl/min. For phase-contrast microscopy, lung slice was observed with an inverted microscope with a 20× objective, and images were recorded using a CCD camera and image acquisition software (Video Savant). Digital images were recorded in time lapse (0.5 Hz) and stored on hard drive. The area of the airway and arteriole lumen was calculated, with respect to time, by pixel summing. Experiments were performed at room temperature.

Measurements of Intracellular Ca^{2+}

Slices were incubated in 20 μM Oregon green 488 BAPTA-1, AM, 100 μM sulfobromophthalein (an inhibitor used to prevent dye extrusion through anion exchangers) and 0.2% pluronic F-127 for 30 min at 30°C followed by 30 min at room temperature and by an additional hour at room temperature in sHBSS containing 100 μM sulfobromophthalein. Lung slices were mounted in the perfusion chamber as described. Fluorescence imaging was performed using a video-rate confocal microscope (Sanderson and Parker, 2003; Sanderson, 2004). In brief, a 488-nm laser was used as the excitation wavelength. The resultant fluorescence (>510 nm) was detected by a photomultiplier tube and a frame capture board to form an image that was recorded to a hard disk. Most recordings were made in time lapse at 2 Hz. Changes in fluorescence intensity were analyzed by selecting regions of interest (ROIs) of ~5 × 5 pixels. 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. Final fluorescence values were expressed as a fluorescence ratio (F_t/F_0) normalized to the initial fluorescence (F_0). For the higher speed recordings of Ca^{2+} waves and elemental Ca^{2+} 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. Paired Student's *t* tests were used to test for significant differences between means. All statistical values are expressed as mean ± SEM.

Online Supplemental Material

Videos (Videos 1–3, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1) consisting of sequences of phase-contrast or fluorescence images were produced with Video Savant. Labels indicate the experimental conditions and the playback speed.

RESULTS

Characteristics and Morphology of Lung Slices

Because the intrapulmonary arteries are anatomically associated with the airways and follow a parallel course through the lungs, an airway and arteriole were readily identified in a lung slice (Fig. 1 A). A detailed description of the morphology of the slice preparation is given in a previous paper (Perez and Sanderson, 2005, Fig. 1). In lung slices, only the alveoli remain filled with agarose; the gelatin was removed from the arterioles by dissolving it at 37°C.

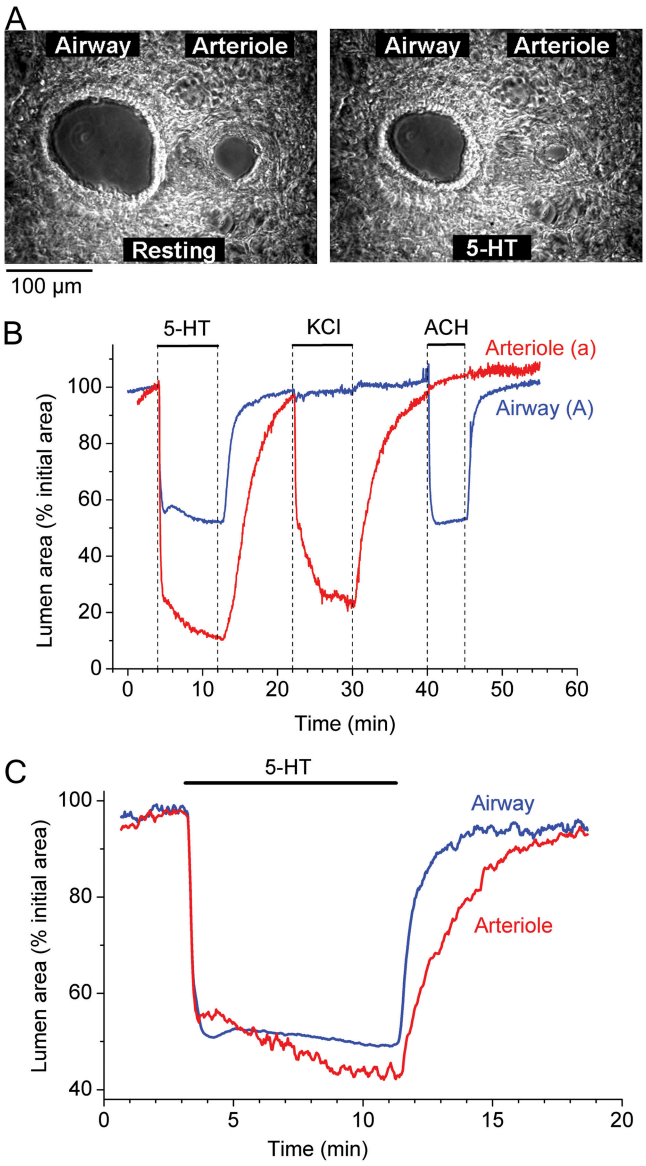

FIGURE 1. The contractile responses of an airway and an arteriole in a lung slice induced by 5-HT, KCl, and ACH. (A) Phase-contrast images showing the appearance of an intrapulmonary small airway (A) and arteriole (a) before stimulation (resting) and 8 min after stimulation with 5-HT. (B) The change in the cross-sectional area of the lumen of an airway (blue line) and arteriole (red line) with respect to time in response to sequential stimulation with 1 µM 5-HT, 100 mM KCl, and 1 µM ACH (black bars): 5-HT induced a large contraction of the arteriole and airway; KCl induced twitching in the arteriole and airway with a sustained contraction in the arteriole but not the airway; ACH induced contraction of the airway but not the arteriole. Upon washing with sHBSS, the arteriole relaxed more slowly than the airway. Representative data selected from six different slices from at least three mice. (C) A comparison of the relaxation rate of an airway (blue) and arteriole (red) from different lung slices but with a similar reduction in the area in response to 1 µM 5-HT (top bar). Although, the initial airway and arteriole contraction rates were similar, the arteriole continued to contract slowly during the presence of 5-HT. The airway relaxed faster than the arteriole upon 5-HT removal. Representative data selected from three slices of three different mice. A movie of these data is shown in Video 1, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1.

The Contractile Response of Arteriole–Bronchiole pairs to 5-HT, KCl, and ACH

The contractile response of arterioles and bronchioles to 5-HT, high KCl, and ACH were initially compared. In response to 1 µM 5-HT, the arteriole quickly contracted (Fig. 1, A and B, red line; Video 1, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1), and this was followed by a slower contraction that reduced the arteriole size by 75 ± 6% after 5 min (8 slices from 3 mice). In response to 1 µM 5-HT, the airway reduced its lumen area by 41 ± 5% after 5 min (Fig. 1, A and B, blue line). Although the initial contraction rates induced by 5-HT in the arteriole and airway were similar, it is important to note that upon 5-HT removal, the relaxation of the arteriole occurred more slowly than that of the airway (Fig. 1, B and C). This differential relaxation rate appears to be related to the SMC type because it was evident at different sizes of contraction and was not correlated with the agonist inducing contraction (5-HT and ACH for airways or 5-HT and KCl for arterioles, see below). However, these differences in relaxation were not observed in response to a transient contraction induced by caffeine (6 slices from 3 mice). In response to 100 mM KCl, the arteriole displayed a large contraction (64 ± 8%) while the airway displayed only a small contraction (14 ± 3%, after 5 min, n = 5 slices from 3 mice). In each case, these contractions were irregular or spasmodic (Video 1). This was especially evident in the airway where the SMCs displayed uncoordinated twitching (Fig. 1 B, blue line). Removal of the KCl, again, resulted in a slow rate of arteriole relaxation. Perfusion with 1 µM ACH had no effect on the arteriole but induced the airway to contract by 57 ± 7% within 5 min (n = 8 slices from 3 mice) (Fig. 1 B; Video 1). Although the arterioles could be deformed by the contraction of the neighboring airway, this stretching did not induce arteriole SMC contraction. Perfusion of lung slices that had been precontacted (with 1 µM 5-HT) with 1 µM ACH did not alter arteriole contraction, but did result in a further contraction of the airway.

From these results, it is clear that the SMCs of arterioles and airways respond differently to different agonists and that the arterioles are not responsive to stretch. Because we characterized the response of the airways in a preceding paper, we focus here on characterizing the contractile and underling Ca^{2+} responses of arteriole SMCs to 5-HT and KCl and compare them to the responses of the airways.

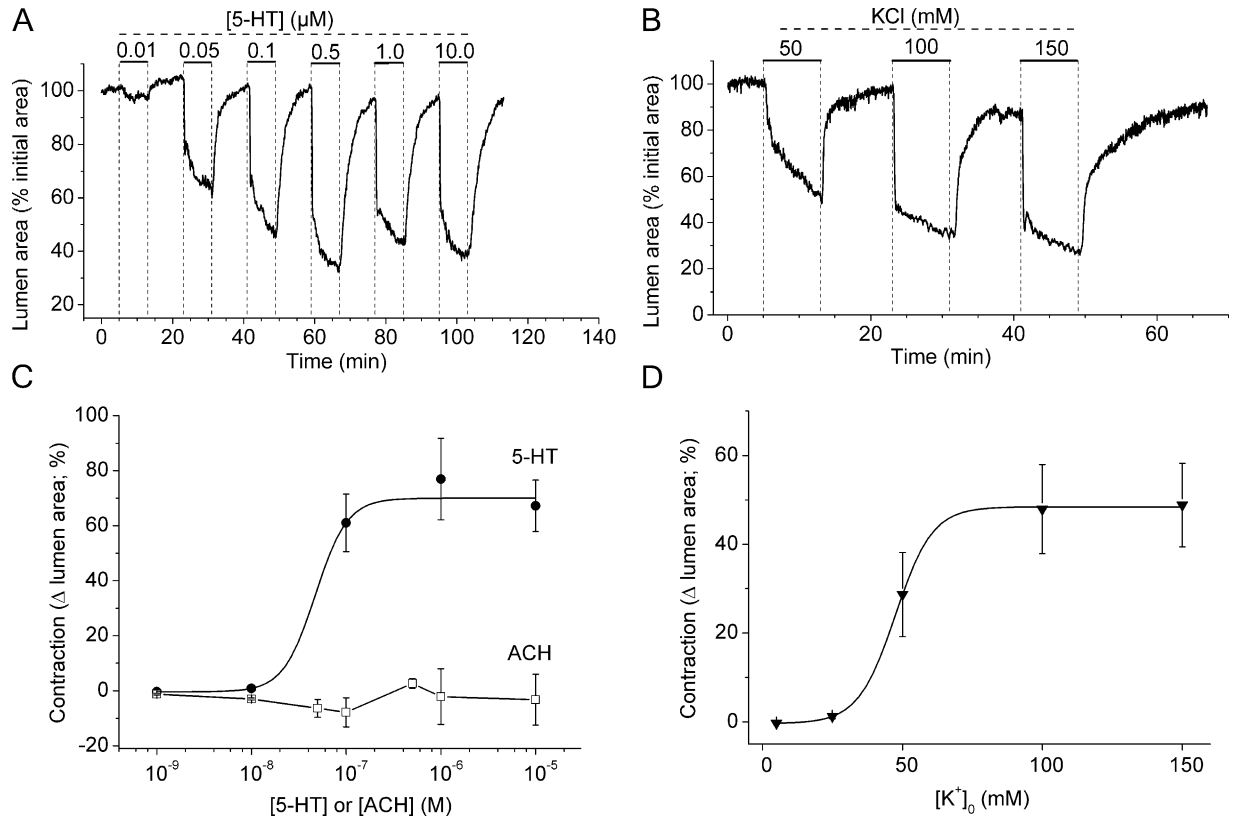

FIGURE 2. The concentration dependence of 5-HT, ACH, and KCl-induced arteriole contraction. (A) 5-HT induced a concentration-dependent increase in the contraction of arterioles in the range of 0.01–0.5 μM. Relaxation of the arterioles between agonist applications was achieved by washing with sHBSS for 10 min. An image was recorded every 2 s and the arteriole lumen cross-sectional area was calculated and plotted with respect to time. Similar experiments performed using (B) isotonic KCl. Summary of the concentration-dependent contractility of the arterioles to (C) 5-HT (closed circles) and ACH (open squares) and (D) KCl. ACH did not contract arterioles at any concentration. Each point is the mean ± SEM from at least three different experiments on different slices from at least two mice. For 5-HT and KCl, the data were fitted with a logistic function curve; the estimated effective concentration (EC_{50}) was 47 nM. ACH data points were joined by straight lines.

Concentration Dependence of Arteriole Contraction to 5-HT and KCl

Increasing concentrations of 5-HT (from 0.01 to 0.5 μM) induced an increasing reduction in luminal size (Fig. 2, A and C). 1 μM 5-HT induced a maximal contraction of ~77 ± 15% (n = 5 from 3 mice). ACH did not induce contraction in arterioles at any concentration tested (Fig. 2 C, n = 6 from 3 mice), even though stimulation of the same arteriole with 5-HT induced ~80% reduction in size (n = 4 from 3 mice). Exposure to increasing concentrations of KCl (from 25 to 100 mM) induced increasing reductions in arteriole area (Fig. 2, B and D). At 100 mM KCl, a maximal contraction of 48 ± 4% (n = 4 from 3 mice) was achieved.

Type of 5-HT Receptor Involved in Arteriole Contraction

Because 5-HT_2 and 5-HT_3 receptors are coupled to increases in $[Ca^{2+}]_i$, antagonists and agonists for these receptors were used to identify the receptor type mediating arteriole contraction. Ketanserin, a 5-HT_2–specific antagonist, at 10^{-8} M completely blocked the contraction induced by 5-HT (3 arterioles from 2 mice). In addition, the sequential stimulation with 10^{-8} to 10^{-6} M DOI (2, 5-dimethoxy-4-iodoamphetamine hydrochloride), a 5-HT_2 specific agonist, induced a concentration-dependent arteriole contraction (3 arterioles from 2 mice). However, SR 57227 (4-amino-1-(6-chloro-2-pyridyl)-piperidine hydrochloride), a 5-HT_3 agonist, at concentrations ranging from 10^{-7} through 10^{-5} M, did not induce contraction, although control stimulations with 5-HT induced contraction of the same arterioles (3 arterioles from 2 mice). These results suggest that the 5-HT_2 receptor is mediating 5-HT responses in intrapulmonary arterioles.

Direct Action of KCl on Arteriole SMCs

To determine if KCl-induced arteriole contraction acts directly or indirectly by stimulating the release of 5-HT or nor-epinephrine from nerve terminals, we studied the effects of ketanserin (a 5-HT_2 antagonist)

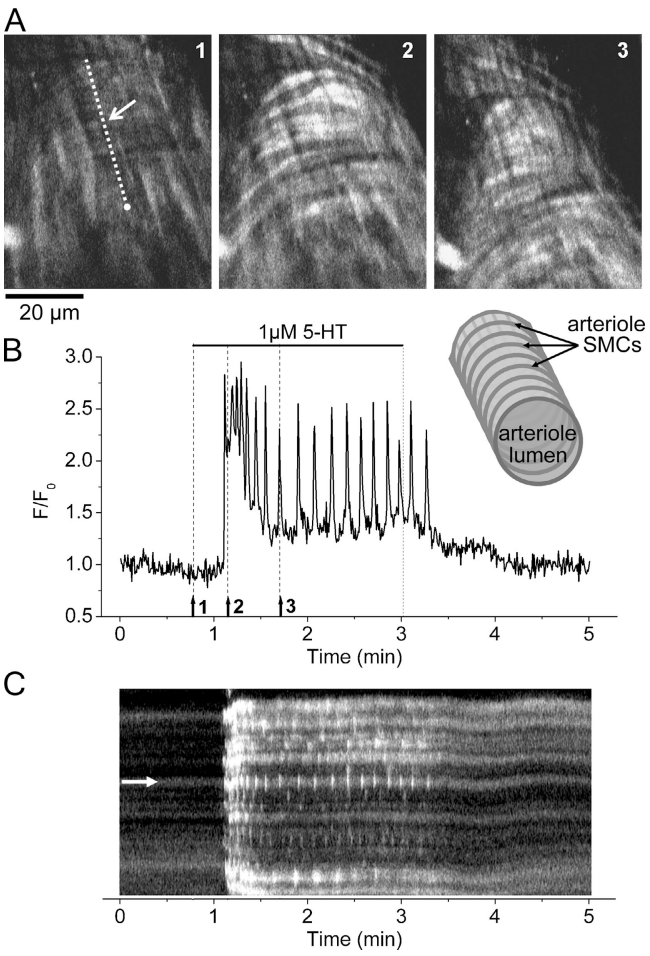

FIGURE 3. Ca^{2+} signaling induced by 5-HT in arteriole SMCs. An arteriole with an oblique orientation (illustrated by the inset drawing) displaying numerous SMCs is shown. (A) Selected images recorded at times indicated in (B) by arrows and dashed line. After stimulation with 1 μM 5-HT (images 2 and 3) the fluorescence in each SMC increased as the arteriole contracted. (B) A small ROI (~5 × 5 pixels) was defined in the SMC indicated by arrow in A, and the fluorescence changes were plotted as a fluorescence ratio (F_t/F_0) with respect to time. The Ca^{2+} signaling induced by 5-HT in the arteriole SMCs was characterized by an initial increase followed by Ca^{2+} oscillations. (C) A line-scan plot with respect to time, from the dotted line indicated in A, showing the Ca^{2+} oscillations (white lines) in different SMCs occurring at different frequencies. Arrow indicates the corresponding SMC of trace in B. Representative data from at least seven trials from different slices from four mice. A movie of the effect of 5-HT on Ca^{2+} signaling in arteriole SMCs is shown in Video 2, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1.

and prazosin (an α_1-adrenoreceptor antagonist) on contraction induced by KCl. Neither ketanserin nor prazosin had any effect on the contraction induced by KCl (3 arterioles from 2 mice). Similarly, neither ACH nor phenylephrine (PE) induced arteriole contraction. These results suggest that KCl acts directly on SMCs and not via the release of ACH, 5-HT, or norepinephrine.

Ca^{2+} Signaling in Arteriole SMCs Induced by 5-HT

In response to 1 μM 5-HT, the arteriole SMCs responded with an increase in $[Ca^{2+}]_i$ followed by the onset of oscillations in $[Ca^{2+}]_i$ (Fig. 3, A and B). These Ca^{2+} oscillations occurred asynchronously, with each cell displaying repetitive increases in $[Ca^{2+}]_i$ at different times with respect to neighboring SMCs (Fig. 3 C). Individual Ca^{2+} oscillations occurred as waves propagating throughout the length of the arteriole SMCs (Video 2, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1; Fig. 9). The increase in $[Ca^{2+}]_i$ in the SMCs was accompanied by the contraction of the arteriole (Fig. 3 A; Video 2).

In the range of 0.01 to 1 μM, 5-HT induced a concentration-dependent increase in the frequency of the Ca^{2+} oscillations in arteriole SMCs (Fig. 4). At 0.01 μM, 5-HT induced a frequency of 0.6 ± 0.2 cycles min^{-1} (n = 7 cells from 3 slices from 2 mice) while at 1 μM, 5-HT induced a frequency of 6 ± 0.5 cycles min^{-1} (n = 13 cells from 6 slices from 3 mice) (Fig. 4 G).

Ca^{2+} Signaling in Arteriole SMCs Induced by KCl

High concentrations of KCl also induced increases in $[Ca^{2+}]_i$ and Ca^{2+} oscillations in arteriole SMCs (Fig. 5; Video 3, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1). However, the KCl-induced Ca^{2+} changes occurred more slowly than those induced by 5-HT, and an initial transient spike was often absent (Fig. 5 A). The frequency of the Ca^{2+} oscillations induced by 25–100 mM KCl was low (one to two per minute), and each Ca^{2+} transient had a long duration (19.1 ± 6.2 s). The KCl-induced Ca^{2+} oscillations of neighboring cells were asynchronous and could occur at different frequencies (Fig. 5 B). The sequential stimulation of the same slice with 1 μM 5-HT induced Ca^{2+} oscillations with a frequency (6.5 ± 0.3 cycles min^{-1}, n = 4, 2 slices from 2 mice) that was significantly higher than those induced by 100 mM KCl in the same SMC (1.2 ± 0.2 cycles min^{-1}; n = 5, 5 slices from 3 mice; $P < 0.05$, t test). However, there were no significant differences in the magnitude of the $[Ca^{2+}]_i$ increases. Similar results were attained when the order of 5-HT and KCl application was reversed ($P < 0.05$, t test, n = 4 cells from 2 slices from 2 mice).

Role of Extracellular Ca^{2+} in SMC Contraction and Ca^{2+} Signaling

In the absence of extracellular Ca^{2+}, 5-HT induced contraction of the arteriole to the same extent as in the presence of extracellular Ca^{2+} (Fig. 6 A; $P < 0.01$, calculated at 1 min after stimulation, n = 5 from 3 mice). In addition, the Ca^{2+} channel blockers $NiCl_2$ or nifedipine were unable to inhibit the onset of the contraction induced by 5-HT (Fig. 6, B and C). By contrast, the

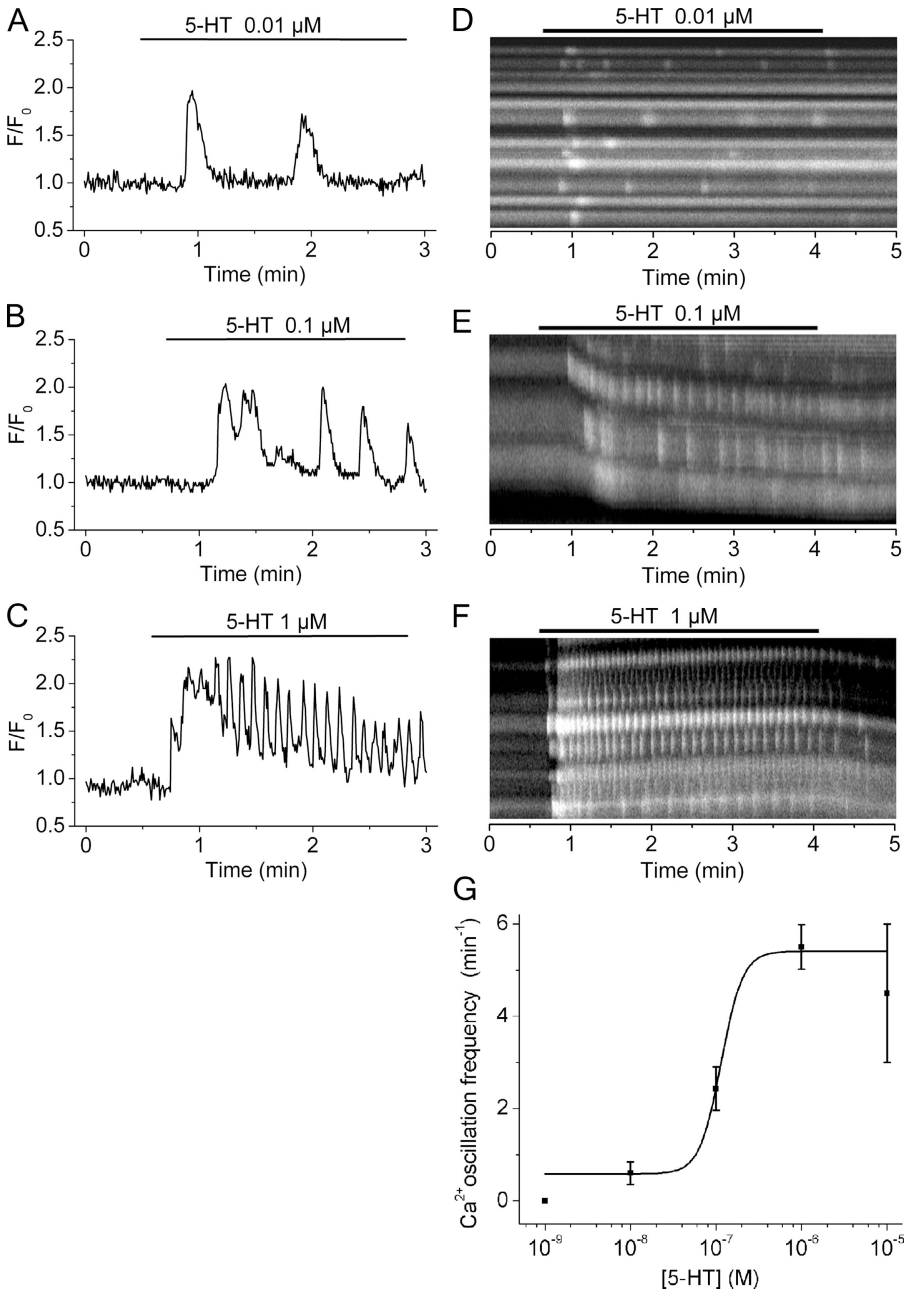

FIGURE 4. Concentration dependence of 5-HT–induced Ca^{2+} signaling in arteriole SMCs. Different lung slices were stimulated with different concentrations of 5-HT as indicated by the bars. (A–C) Representative changes in fluorescence in defined ROIs over individual SMCs for each concentration. Increases in 5-HT concentration induced increases in the frequency of Ca^{2+} oscillations. (D–F) Line-scan plots showing the responses to different concentrations of 5-HT in adjacent SMCs. (G) The mean frequency of Ca^{2+} oscillations induced in arteriole SMCs by 5-HT; mean ± SEM of at least seven cells from slices from three mice. **, $P < 0.01$ between all concentrations (Student's *t* test).

absence of extracellular Ca^{2+} or the presence of $NiCl_2$ or nifedipine completely abolished the contraction induced by KCl (Fig. 6, D–F). While effect of Ni^{2+} was reversible, the effect of nifedipine was not, a result consistent with a high affinity binding site for 1,4-dihydropyridines to transmembrane segments of the α1 subunits of L-type Ca^{2+} channels (Striessnig et al., 1998). These results indicate that extracellular Ca^{2+} or Ca^{2+} influx was not necessary to trigger the contraction induced by 5-HT, but was required to mediate contraction induced by KCl. However, after 3 min of 5-HT stimulation, in the absence of external Ca^{2+} or presence of $NiCl_2$ or nifedipine, the normal secondary slow rate of contraction was arrested and, in some cases, the arteriole began to relax (Fig. 6, A–C).

In both the presence or absence of extracellular Ca^{2+}, 5-HT induced an initial increase in $[Ca^{2+}]_i$ followed by Ca^{2+} oscillations (Fig. 7). Although the Ca^{2+} oscillations persisted with an approximately constant frequency during the presence of the agonist and extracellular Ca^{2+} (Fig. 7, A and B), the oscillations slowed and ceased within 3 min in the absence of external Ca^{2+} (Fig. 7, C and D).

The ability of 5-HT to induce Ca^{2+} oscillations in the absence of external Ca^{2+} correlates with its capacity to initiate arteriole contraction in the absence of external

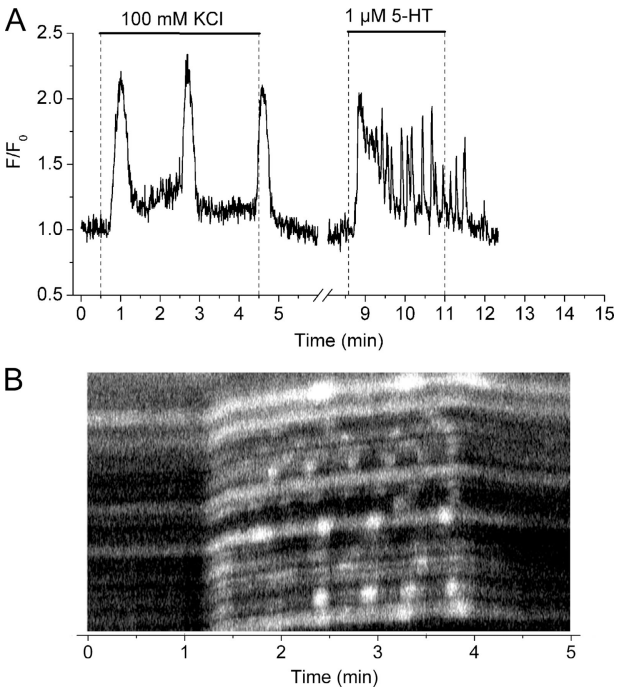

FIGURE 5. Ca^{2+} signaling induced by 100 mM KCl in arteriole SMCs. (A) Changes in fluorescence determined from an ROI within a single SMC showing slow Ca^{2+} oscillations induced initially by 100 mM KCl and higher frequency Ca^{2+} oscillations subsequently induced with 1 μM 5-HT. Traces are representative of at least three SMCs from different slices from three mice. (B) A line-scan plot showing the response in several adjacent SMCs in an arteriole. Cells displayed slow Ca^{2+} oscillations induced by KCl (white lines). Representative data from six experiments from slices from three mice. A movie showing the effects of KCl on Ca^{2+} signaling in arteriole SMCs is shown in Video 3, available at http://www.jgp.org/cgi/content/full/jgp.200409217/DC1.

Ca^{2+} (Fig. 6 A). Similarly, the inhibition of the Ca^{2+} oscillations after 3 min in the absence of Ca^{2+} correlates with the inhibition of the slow-rate contraction and the initiation of relaxation of the arteriole. In the absence of extracellular Ca^{2+}, KCl did not stimulate any change in the $[Ca^{2+}]_i$ ($n = 3$ arterioles from 2 mice), a result that correlates with the inability of KCl to induce arteriole contraction under these conditions (Fig. 6 D).

Effect of Caffeine on Arteriole Contraction Induced by 5-HT and KCl

To characterize the contribution of the intracellular Ca^{2+} stores and RyRs in the contraction of arterioles, we examined the effects of caffeine on the contractile response induced by 5-HT and KCl. In arteriole SMCs, caffeine induced a transient increase in $[Ca^{2+}]_i$ that subsided to a sustained elevation in Ca^{2+}, which itself returned to the prestimulatory level after the caffeine was removed (Fig. 8 A). The sustained elevation of Ca^{2+} was quickly abolished when extracellular Ca^{2+} was withdrawn (Fig. 8 B). In the absence of extracellular Ca^{2+}, caffeine induced a transient Ca^{2+} increase without a sustained plateau (Fig. 8 C). However the readdition of external Ca^{2+} resulted in a sustained increase in Ca^{2+} (Fig. 8 C). In response to repeated exposures to caffeine, arterioles elicited single transient contractions that quickly relaxed to the prestimulatory state within a minute (Fig. 8 D). The response to both 5-HT and KCl was reversibly abolished by caffeine (Fig. 8, E and D). These results indicate that caffeine stimulates the release of Ca^{2+} from internal stores and stimulates a Ca^{2+} influx across the plasma membrane of arteriole SMCs. However, the sustained elevation of Ca^{2+} induced by caffeine does not appear to directly participate in the contractile response of arterioles. While these results are compatible with the expected result that a release of intracellular Ca^{2+} was necessary to trigger and sustain the contraction induced by 5-HT, they also highlight a significant dependence of the effects of KCl on internal stores.

Ca^{2+} Waves and Elemental Ca^{2+} Events in Arteriole SMCs Induced by 5-HT and KCl

To compare the spatio-temporal properties of the Ca^{2+} oscillations induced by 5-HT and KCl in arteriole SMCs, fast (60 fps) recordings of single SMCs were performed during continuous stimulation with each agonist. For analysis, line-scan plots from the longitudinal axes of single SMCs were made; these show approximately evenly spaced diagonal lines that represent the Ca^{2+} oscillations induced by 5-HT that consist of repetitive Ca^{2+} waves which propagated along the longitudinal axes of the cell (Fig. 9). While the wave velocity (slope of the lines) could change in magnitude and sometimes in direction, the average 5-HT–induced Ca^{2+} wave velocity was 21 ± 3 μm/s. No other changes in $[Ca^{2+}]_i$ were observed in the period between two successive Ca^{2+} waves.

By contrast, KCl-induced Ca^{2+} oscillations appeared as longer lasting Ca^{2+} waves or tides. However, the mean wave propagation velocity (16 ± 3 μm/s) was similar to that observed with 5-HT. A unique feature of KCl-induced Ca^{2+} waves was that they were preceded by small, localized, and transient Ca^{2+} increases (elemental Ca^{2+} events) that did not spread throughout the whole cell (Fig. 9, arrows). These elemental Ca^{2+} events were initiated from one or more specific localizations in a cell with an average frequency of 8 ± 2 per min (5 cells from 3 slices from 2 mice). However the frequency of these events increased up to the time when the next Ca^{2+} wave was initiated. After each Ca^{2+} wave had subsided, a refractory period occurred before the Ca^{2+} events started to occur again. Ca^{2+} waves were usually initiated from one location showing the highest frequency of Ca^{2+} events. Each elemental Ca^{2+} event occurred with a diameter ~4 μm, had a rise-to-peak

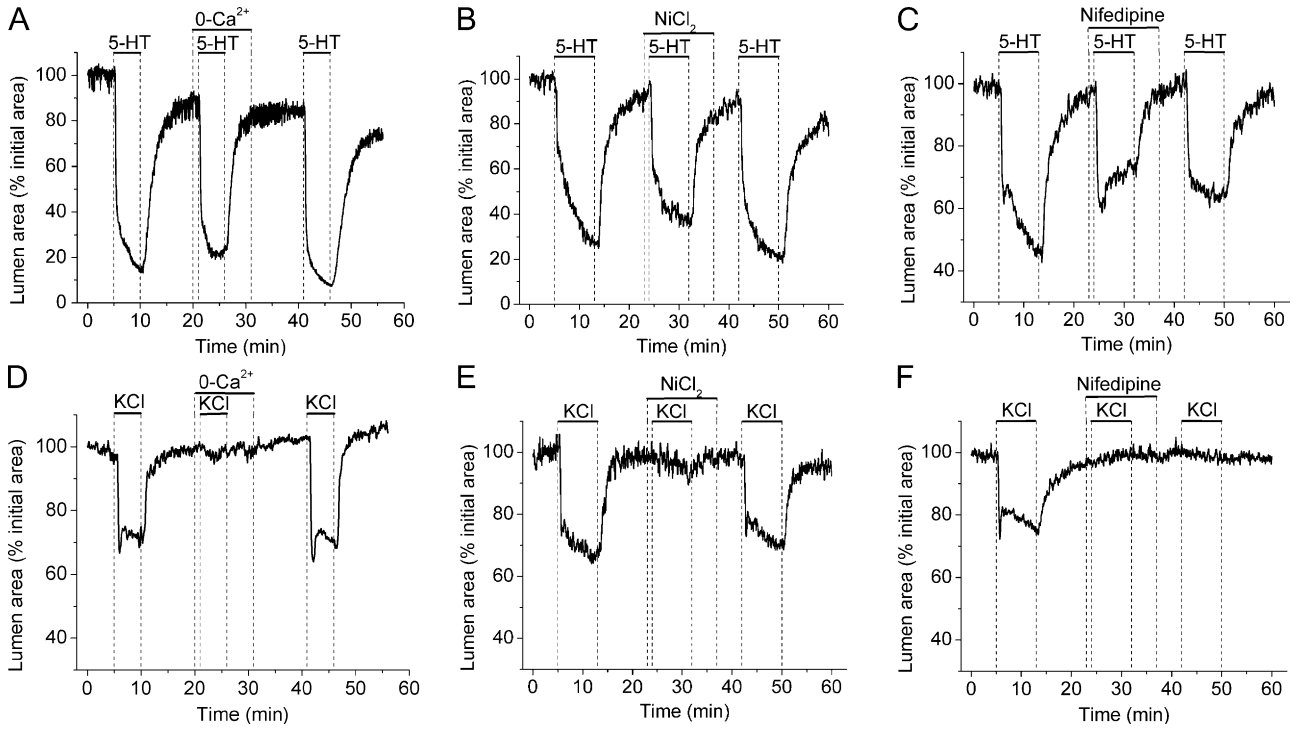

FIGURE 6. The effect of the absence of extracellular Ca^{2+}, nifedipine, or $NiCl_2$ on the contraction of arterioles induced by 5-HT and KCl. Arterioles, in different lung slices, were sequentially exposed (top bars) to 1 μM 5-HT (A–C) and 100 mM KCl (D–F) in the presence or absence of extracellular Ca^{2+} (A and D) and the presence or absence of the Ca^{2+} channel blockers; 1 mM $NiCl_2$ (B and E) or 10 μM nifedipine (C and F). Traces are representative of at least three experiment slices for each condition from different slices from three mice.

time of 70 ± 30 ms, and a first order exponential decay rate of 385 ± 122 ms (Fig. 9, inset a, $n = 4$ cells from 3 slices from 2 mice). Ca^{2+} waves and elemental Ca^{2+} events were not observed during KCl stimulation in the presence of caffeine.

DISCUSSION

We used a lung slice preparation to study the contractile responses of intrapulmonary arterioles and the underlying changes in $[Ca^{2+}]_i$ in their SMCs during stimu-

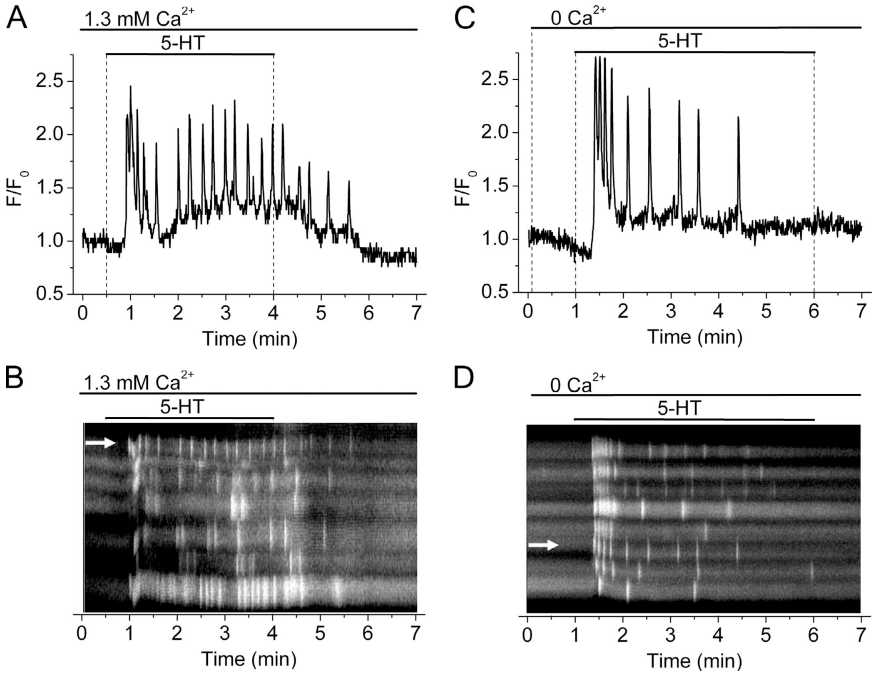

FIGURE 7. Role of extracellular Ca^{2+} in 5-HT–induced Ca^{2+} signaling in arteriole SMCs. Lung slices were stimulated with 1 μM 5-HT in (A and B) the presence or (C and D) absence of extracellular Ca^{2+} as indicated by bars. (A and C) Representative changes in fluorescence in defined ROIs over individual SMCs for each condition. (B and D) Line-scan plots showing the Ca^{2+} oscillations (white lines) in several adjacent SMCs. 5-HT induced an increase in $[Ca^{2+}]_i$ followed by Ca^{2+} oscillations that persisted in the presence of extracellular Ca^{2+} but stopped in absence of extracellular Ca^{2+}. Representative traces of at least four experiments in different slices from three different mice.

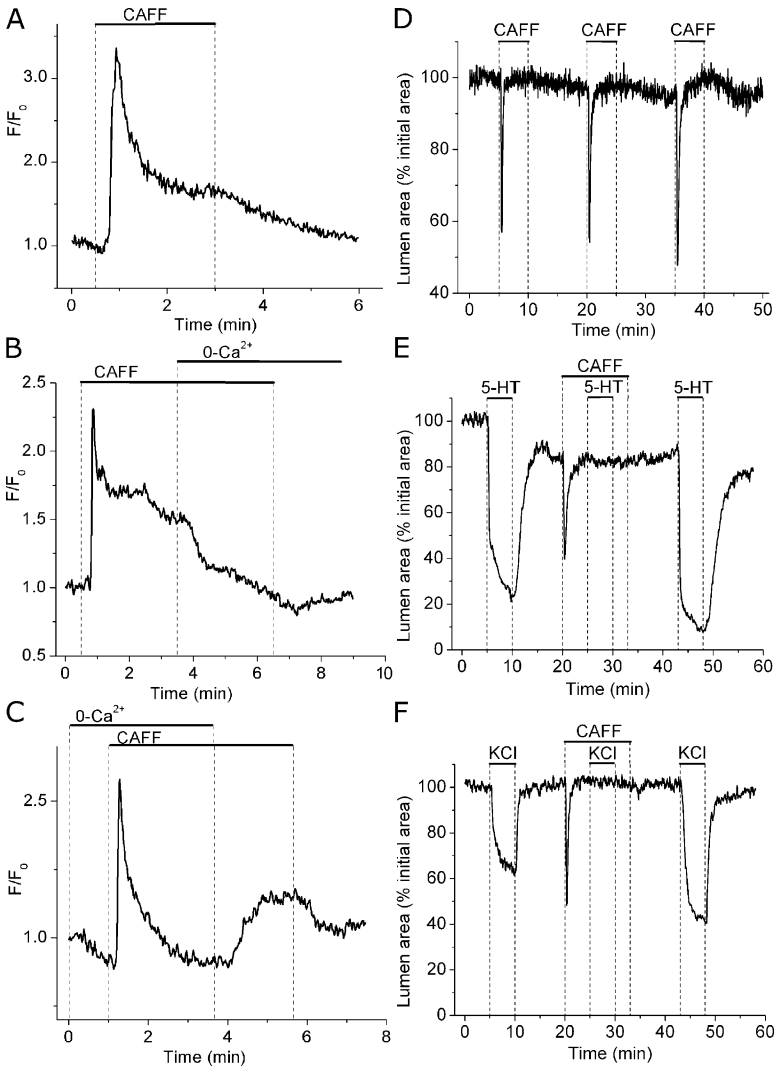

FIGURE 8. The effect of caffeine on the Ca^{2+} signaling of SMCs and contraction of arterioles induced by 5-HT and KCl. (A–C) Changes in $[Ca^{2+}]_i$ in individual arteriole SMCs induced by 20 mM caffeine in the presence or absence of extracellular Ca^{2+} as indicated by bars. (D) Transient contractions in arterioles in response to repetitive exposures of 20 mM caffeine as indicated by bars. (E and F) Lung slices were stimulated with (E) 1 μM 5-HT or (F) 100 mM KCl in the absence or presence of 20 mM caffeine as indicated by the bars. Representative traces of at least three experiments in different slices from two different mice.

lation with 5-HT and KCl. For the same reasons that apply to the study of bronchial airways (Bergner and Sanderson, 2002a), thin lung slices are well suited for the study of arterioles. The arterioles are easily identified, have reproducible contractile responses and the intracellular Ca^{2+} responses of their SMCs can be correlated with the contraction of the arteriole. In addition, the small intrapulmonary arterioles, at sites that are considered to be important in pulmonary hypertension, can be examined. A major advantage of the lung slice is the ability to simultaneously compare the responses of arteriole SMCs to those of airway SMCs. This allows for the instant collection of control data and facilitates an understanding of the specific physiological responses of each SMC type.

Vasoconstriction of pulmonary arteries to 5-HT has been observed in most animal species, including mice and humans (MacLean et al., 2000). In mouse lung slices, we found that 5-HT induced a concentration-dependent contraction of arterioles over the range of 0.01–1 μM with an EC_{50} of ~50 nM. A similar sensitivity to 5-HT was observed in intrapulmonary artery rings from mice (EC_{50} = 80 nM) (Liu and Folz, 2004). In humans, the sensitivity of intrapulmonary arteries is reported to be slightly lower in intact (EC_{50} = 257 nM; MacLean et al., 1996) or endothelium-denuded intrapulmonary rings (390 nM; Cortijo et al., 1997). Differences in the sensitivity to 5-HT of intrapulmonary arteries between the mouse and human could be due to differences in the type(s) of 5-HT receptor expressed in each species.

We found here that arteriole contraction stimulated by 5-HT was associated with an initial increase in $[Ca^{2+}]_i$ and fast Ca^{2+} oscillations followed by a period where the Ca^{2+} oscillations persisted with a slower but more constant frequency. Like the Ca^{2+} oscillations of airway SMCs, the oscillations spread along the cell as Ca^{2+} waves. The Ca^{2+} oscillations most probably result from repetitive cycles of Ca^{2+} release and reuptake by intracellular stores because Ca^{2+} oscillations could be

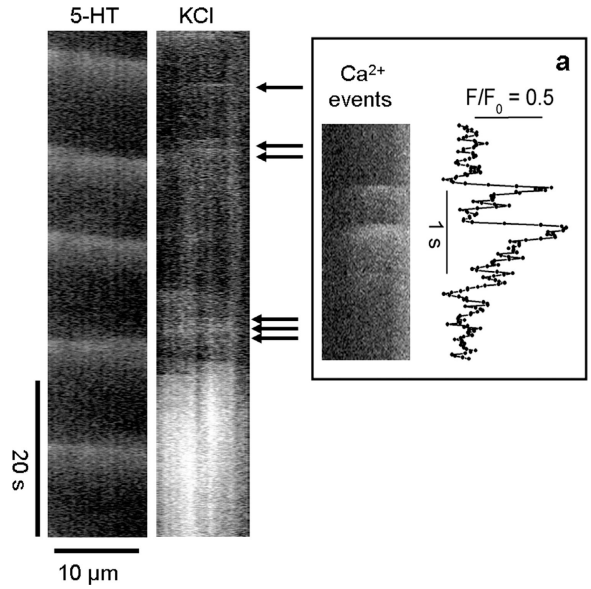

FIGURE 9. Ca^{2+} waves and single Ca^{2+} events in arteriole SMCs during stimulation with 5-HT and KCl. Line-scan plots from the longitudinal axes of single arteriole SMCs from high speed recordings (60 fps) of changes in Ca^{2+} during continuous perfusion with 1 μM 5-HT and 50 mM KCl as indicated. Note that the slopes of the bright diagonal lines indicate the velocity (μm/s) and direction of the Ca^{2+} waves in the SMCs. KCl induces small Ca^{2+} events (arrows) preceding the lower Ca^{2+} wave. Two of such Ca^{2+} events (image and time trace) induced by KCl are expanded in inset a. Representative data from four experiments from different slices of two mice.

initiated in absence of extracellular Ca^{2+} or in presence of Ca^{2+} channel blockers. However, the eventual cessation of the Ca^{2+} oscillations in the absence of extracellular Ca^{2+} suggests a need for some extracellular Ca^{2+} influx.

In view of the robust Ca^{2+} oscillation that we observed in intrapulmonary arterioles, it is surprising that Ca^{2+} oscillations were not observed by Guibert et al. (2004), who investigated 5-HT–induced Ca^{2+} signaling in segments of cannulated and pressurized rat intrapulmonary arteries. A reason for this difference, as the authors conceded, was probably the low spatio-temporal resolution of the Ca^{2+} measurements. Wide-field microscopy was used to observe a multicellular tissue with a sampling rate of one image/20 s. It is likely that the small increases in $[Ca^{2+}]_i$ induced by increasing concentrations of 5-HT that correlated with contraction (Guibert et al., 2004) resulted from an averaging of increasing frequencies of asynchronous Ca^{2+} oscillation in multiple SMCs. Therefore, the interpretation that a sustained contraction of SMCs results from a sustained increase in $[Ca^{2+}]_i$ induced by a constant influx of Ca^{2+} through receptor-operated channels should be reevaluated. Because pharmacological evidence supported the existence of receptor-operated channels in intrapulmonary arteries (Guibert et al., 2004), we suggest that these may be involved in refilling Ca^{2+} store to maintain the Ca^{2+} oscillations. The reliance on Ca^{2+} oscillations rather than sustained increases in $[Ca^{2+}]_i$ to maintain contraction is also a way to avoid toxic effects of elevated $[Ca^{2+}]_i$ (Savineau and Marthan, 2000; Lee et al., 2002).

The Ca^{2+} oscillations induced by 5-HT in lung arterioles were asynchronous between neighboring cells at all 5-HT concentrations. A similar finding is reported for rat mesenteric artery with PE-induced Ca^{2+} oscillations (Mauban et al., 2001). These observations are different to those reported for rat mesenteric arteries (Lamboley et al., 2003) where synchronization of the Ca^{2+} oscillations and recruitment of SMCs showing Ca^{2+} oscillations was observed with increasing concentrations of the PE. In contrast to our observations of both airway and arteriole SMCs, the sustained contraction of mesenteric arteries was observed only at high concentrations of PE and correlated with the recruitment and the synchronization of the Ca^{2+} oscillations (Lamboley et al., 2003). We have not observed any synchronization or substantial recruitment of cells with increasing agonist concentrations in either arteriole or airway SMCs. It is important to note that in lung arteriole and airway SMCs, as well as in rat mesenteric arteries (Mauban et al., 2001), each Ca^{2+} oscillation observed during 5-HT stimulation did not generate a transient contraction, but that the SMCs maintained a steady contractile state at all concentrations of 5-HT. This has the important implication that the contractile elements of the cell serve to integrate the stimuli encoded in the Ca^{2+} oscillations. Although, it has been reported that SMCs of a number of tissues, including airways (Kuo et al., 2003) and blood vessels (Christ et al., 1996), are electrically coupled, we have not observed Ca^{2+} waves spreading to adjacent cells in arterioles or airways. One explanation for this is that the concentration of inositol 1,4,5-trisphosphate (IP_3) (a permeable messenger) would be expected to be similar in neighboring cells during global stimulation with the agonists.

In similarity to airway SMCs, the frequency of the Ca^{2+} oscillations and the extent of contraction induced by 5-HT in arterioles was concentration dependent. However, the frequency of the Ca^{2+} oscillations observed in airways was, in general, higher (~20 or 30 cycles/min with maximal concentrations of 5-HT or ACH, respectively) than those observed in arterioles during 5-HT stimulation (approximately six cycles/min with maximal concentrations). Other systemic blood vessels have also been observed to have a concentration-dependent increase in contraction and Ca^{2+} oscillation frequency when stimulated with a variety of agonists (Lee et al., 2002). For example, in the rabbit inferior vena cava, PE ranging from 0.15 to 150 μM

increased the frequency of Ca^{2+} oscillations from 3 to 30 cycles/min and the extent of contraction (Ruehlmann et al., 2000). PE was also found to stimulate a concentration-dependent increase in Ca^{2+} oscillations and contraction in rat mesenteric arteries (Mauban et al., 2001; Lamboley et al., 2003). This correlation between contraction and Ca^{2+} oscillation frequency suggests that the size of arteriole contraction is regulated by frequency modulation (FM) of the $[Ca^{2+}]_i$ (Berridge et al., 2003) in a similar manner to that found for airway contraction.

The most likely mechanism for 5-HT–induced arteriole contraction and $[Ca^{2+}]_i$ signaling is that 5-HT binds to a 5-HT_2 receptor coupled through Gq protein to PLC (Hoyer et al., 2002). Activation of PLC generates IP_3 to initiate the release of Ca^{2+} from the SR through IP_3 receptors. Persistent oscillations in $[Ca^{2+}]_i$ are produced by continuous cycles of release and reuptake of Ca^{2+} from internal stores. Support for this mechanism is provided by the fact that contraction was inhibited by ketanserin, a 5-HT_2 receptor blocker and stimulated by DOI, a 5-HT_2 receptor agonist. While 5-HT_3 receptors can elevate $[Ca^{2+}]_i$, via a Ca^{2+} influx, 5-HT–induced increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} and the 5-HT_3 receptor agonist SR 57227 did not stimulate the arteriole contraction. It has also been suggested that the 5-HT_{1B}/5-HT_{1D} receptors participate in the contraction of intrapulmonary arteries in humans (MacLean et al., 1996; Cortijo et al., 1997; Morecroft et al., 1999), mouse (Keegan et al., 2001; Liu and Folz, 2004), and other species (Shaw et al., 2000; Murdoch et al., 2003). However, these receptors are coupled to $G_{i/o}$ to produce a decrease in cAMP (Hoyer et al., 2002), and we believe that these receptors are not the major pathway for 5-HT–induced Ca^{2+} oscillations in arteriole SMCs.

While 5-HT appears to be a potent vasoconstrictor of pulmonary arterioles, the arterioles did not contract or relax in response to ACH at any concentration that produced contraction of airway SMCs. This suggests that the cholinergic pathway is not involved in the contractile response of the arterioles. In addition, arterioles precontracted with 5-HT did not relax in response to ACH. In other vascular SMCs, ACH induced relaxation via stimulation of the endothelial cells. However, the failure to observe this response in lung slices with constant perfusion could be explained by the fact that any factors released by endothelial cells would be washed away or by an inability of the cells to respond to ACH. To resolve these issues, further studies of the role of the endothelial cells in lung slices is required.

The mechanism by which KCl triggered Ca^{2+} oscillations in arteriole SMCs was dependent on extracellular Ca^{2+} influx, sensitive to $NiCl_2$ and nifedipine, blocked by caffeine and characterized by Ca^{2+} waves occurring at very low frequencies. In contrast to 5-HT–induced Ca^{2+} waves, KCl-induced Ca^{2+} waves were preceded by small, localized Ca^{2+} increases or elemental Ca^{2+} events. As a result, we believe that KCl-induced membrane depolarization resulted in the activation of voltage-gated Ca^{2+} channels and that this, in turn, led to an influx of Ca^{2+}. However, instead of directly inducing SMC contraction, this additional Ca^{2+} is loaded into the SR. As the Ca^{2+} load within the internal store increases, the chance that Ca^{2+} is released from the SR, through RyRs, is increased. After reaching a critical threshold, the elemental Ca^{2+} release is amplified by CICR to produce a long-lasting Ca^{2+} wave that results in contraction of the SMCs and the arteriole. Ca^{2+} waves induced by KCl did not occur synchronously in adjacent cells, which suggests that they do not result from cycles of depolarization and hyperpolarization in electrical coupled cells. This explanation for KCl-induced Ca^{2+} oscillations in arteriole SMCs is identical for airway SMCs. These responses to KCl also highlights the need for caution when assuming that sustained contraction results directly from increases in Ca^{2+} induced by depolarization.

The alternative hypothesis that KCl acts indirectly via the depolarization of neural terminals to locally release neurotransmitters (Lamont et al., 2003) does not appear to be applicable in lung slices. Most importantly, the arterioles did not respond to PE or ACH. Similarly, prazosin or ketanserin had no effect on the contraction induced by KCl. In addition, our experiments were performed with constant perfusion that would quickly wash away any endogenously released transmitters, yet the KCl effects persisted for longer periods and show similar responses during repetitive stimulations.

Probably, the most important implication of our results is that a sustained contraction of intrapulmonary arteriole SMCs is maintained and regulated by the frequency of Ca^{2+} oscillations rather than by a sustained Ca^{2+} elevation. We have reached an identical conclusion for the regulation of airway SMCs contraction. In addition, other recent studies using confocal Ca^{2+} imaging of nonintrapulmonary blood vessels or airways have observed Ca^{2+} oscillations rather than sustained elevations in Ca^{2+} during stimulation with agonists (Lee et al., 2002; Li et al., 2003; Wier and Morgan, 2003).

An FM regulation hypothesis can explain the contraction of either the arteriole or airway SMCs, but the FM relationship of contraction to Ca^{2+} oscillation frequency is very different between airway and arteriole SMCs. For example, the size of arteriole contraction ($\sim$70%) induced by slow Ca^{2+} oscillations (six per minute) stimulated by 5-HT was often greater than the contraction induced in airways ($\sim$50%) by much faster oscillations (15–20 per minute) with the same agonist

(Perez and Sanderson, 2005). To explain this difference, we hypothesize that the contractile state is determined, not only by the frequency of the oscillations but, also by the relaxation rate of the SMC. In this hypothesis, the rate at which SMCs develop Ca^{2+}-dependent contraction is similar but the rate of relaxation from the contracted state serves to integrate the contractile response. A variation in this rate between SMC types will determine the frequency of the Ca^{2+} oscillations required to sustain contraction; a slow or fast relaxation rate will require low or high frequency Ca^{2+} oscillations, respectively.

The relaxation rate of airway SMCs appears to be faster than that of arteriole SMCs under all conditions. After the removal of 5-HT, the airways relax quicker than the arterioles. While SMC relaxation occurs following the cessation of the Ca^{2+} oscillations, the period between the stoppage of the Ca^{2+} oscillations and relaxation is shorter in airways. Under extracellular Ca^{2+}-free conditions, the Ca^{2+} oscillations of both the airway and arteriole SMC rundown and the airway relax immediately but the arterioles only just began to relax after 5 min. Another major clue is presented by the twitching response of the SMCs in response to slow Ca^{2+} oscillations induced by KCl (Perez and Sanderson, 2005). The contractile state of the airway SMCs closely followed the changes in $[Ca^{2+}]_i$, indicating that the Ca^{2+}-induced contraction can only be maintained for a short duration. By contrast, arteriole SMCs show modest twitching to the KCl-induced Ca^{2+} oscillations and maintained a substantial contraction. Thus, while both airway and arteriole SMCs quickly contract in response to Ca^{2+}, arteriole SMCs sustain longer contractions to each Ca^{2+} pulse than the airway SMCs. Therefore, for airway SMCs to maintain contraction, the time interval between Ca^{2+} pulses must be short; a prediction that matches the fast frequency of Ca^{2+} oscillation observed in airway SMCs (Perez and Sanderson, 2005). Conversely, arterioles only require slow frequency Ca^{2+} oscillations to maintain contraction.

The immediate question raised by this differential relaxation hypothesis is the nature of the difference between the two SMC types. One possibility is that slower Ca^{2+} dynamics result in higher resting Ca^{2+} levels to delay relaxation. However, the base line of the Ca^{2+} oscillation does not seem to be correlated with the contractile state. Similarly, there is little difference between the duration of each Ca^{2+} oscillation or the basal Ca^{2+} level of the Ca^{2+} oscillations induced by KCl in airways and arterioles, yet the relaxation of the airways is faster. Thus, the relaxation time does not seem to be directly related to $[Ca^{2+}]_i$. To explain the different relaxation times, we speculate that the dissociation kinetics of dephosphorylated, but attached, actin–myosin crossbridges that are formed during actin–myosin cycling to produce contraction (Mijailovich et al., 2000) is slower in arteriole SMCs than in airway SMCs. This idea is supported by simulated contractions predicted by mathematical modeling of crossbridge formation in a Hai-Murphy four-state model (Hai and Murphy, 1989) in response to Ca^{2+} oscillations, but this idea will require further investigation.

In conclusion, intrapulmonary arterioles respond to 5-HT with a sustained contraction that is maintained by persistent and asynchronous Ca^{2+} oscillations of the associated SMCs. These Ca^{2+} oscillations are generated and propagated as waves by repetitive cycles of Ca^{2+} release and reuptake by the SR and require extracellular Ca^{2+} for store refilling. The size of the contraction of arterioles is regulated by the frequency of the Ca^{2+} oscillations in SMCs rather than by the amplitude of a sustained $[Ca^{2+}]_i$ increase. We hypothesize that the relaxation kinetics of the SMC serves to integrate the Ca^{2+}-dependent contractile response of the SMC. One implication of this hypothesis is that hyperactivity can result from a change in the relaxation kinetics of the SMC as well as from a change in the Ca^{2+} signaling of the SMC.

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