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Histamine-mediated Increases in Cytosolic $[Ca^{2+}]$ Involve Different Mechanisms in Human Pulmonary Artery Smooth Muscle and Endothelial Cells

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Abstract

Agonist stimulation of human pulmonary artery smooth muscle (PASMC) and endothelial (PAEC) cells with histamine showed similar spatiotemporal patterns of Ca^{2+} release. Both sustained elevation and oscillatory patterns of changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) were observed in the absence of extracellular Ca^{2+} . Capacitative Ca^{2+} entry (CCE) was induced in PASMC and PAEC by passive depletion of intracellular Ca^{2+} stores with 10- μ M cyclopiazonic acid (CPA, for 15-30 min). The pyrazole derivative BTP2 inhibited the CPA-activated Ca^{2+} influx, suggesting that depletion of CPA-sensitive internal stores is sufficient to induce CCE in both PASMC and PAEC. The recourse of histamine-mediated Ca^{2+} release was examined after exposure of the cells to CPA, thapsigargin, caffeine, ryanodine, FCCP, or bafilomycin. In PASMC bathed in Ca^{2+} -free solution, treatment with CPA almost abolished histamine-induced rises in $[Ca^{2+}]_{cyt}$. In PAEC bathed in Ca^{2+} -free solution, however, treatment with CPA eliminated the histamine-induced sustained and oscillatory rises in $[Ca^{2+}]_{cyt}$, but did not affect the initial transient increase in $[Ca^{2+}]_{cyt}$. Furthermore, treatment of PAEC with a combination of CPA (or thapsigargin) and caffeine (and ryanodine), FCCP, or bafilomycin did not abolish the histamine-induced transient $[Ca^{2+}]_{cyt}$ increases. These observations indicate that *a*) depletion of CPA-sensitive stores is sufficient to cause CCE in both PASMC and PAEC; *b*) induction of CCE in PAEC does not require depletion of all internal Ca^{2+} stores; *c*) the histamine-releasable internal stores in PASMC are mainly CPA-sensitive stores; *d*) PAEC, in addition to a functional pool that is sensitive to CPA, contain other stores that are insensitive to CPA, thapsigargin, caffeine, ryanodine, FCCP, and bafilomycin; and *e*) the CPA-insensitive stores in PAEC, while may not contribute to CCE, contribute to histamine-mediated Ca^{2+} release.

Keywords

histamine; intracellular calcium stores; oscillations; pulmonary hypertension

Introduction

The regulation of pulmonary vascular tone determines the regional blood perfusion and optimal ventilation-perfusion ratio in the lung, which are required for normal gas exchange. Hypoxia-induced pulmonary vasoconstriction, for example, directs blood flow away from the poorly ventilated areas to achieve maximal oxygenation of the venous blood in the pulmonary artery (62,67). The contractile state of pulmonary arteries is influenced by neural, humoral, and endothelial factors (4). Agonist-mediated changes in cytoplasmic Ca^{2+} concentration

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($[Ca^{2+}]_{cyt}$) in pulmonary artery smooth muscle (PASM) (19,44,51,66) and endothelial (PAEC) (25,27) cells play a pivotal role in the regulation of excitation-contraction coupling in the pulmonary vasculature. The interaction of PAEC with PASM via paracrine agonists, upon activation of receptors in either type of these cells, determines whether vessels constrict or relax in response to certain agonists (4,32). Histamine, a molecule mainly synthesized and released from mast cells in the adventitial layer of pulmonary vessels (e.g., during inflammatory response), is a vasoactive substance with dual-effects on the pulmonary vasculature. Histamine can elicit vasoconstriction or vasodilation depending on the cell type (e.g., smooth muscle cells or endothelial cells) on which it is acting and on the receptor subtypes (e.g., H_1 , H_2 , H_3 or H_4) activated (1,11,28,30,31,50).

Histamine exerts a vasoconstrictive effect, presumably via activation of H_1 receptors, but also exerts a vasodilatory effect on pulmonary arterial muscle preparations (9,39). Whereas the dual effects of histamine on vessel caliber of human pulmonary arteries have been known for some time, the Ca^{2+} signals that underlie stimulation of PASM and PAEC with histamine have not been extensively examined. Previous studies have shown that histamine evokes oscillatory membrane currents via release of Ca^{2+} from internal stores in freshly dissociated rabbit PASM (64) and induces Ca^{2+} release and influx in human umbilical vein endothelial cells (37) and PAEC (47,60). PAEC also show an increase in $[Ca^{2+}]_{cyt}$ in response to hypercapnia, where Ca^{2+} is released from thapsigargin-insensitive stores (38). Relatively higher dose of histamine is known to elicit heparin-inhibitable Ca^{2+} -release (concurrent with significant phosphatidylinositol hydrolysis) in human PAEC (60). The nature (and identity) of the IP_3 -sensitive stores involved in the response to histamine, however, was not closely examined in these previous reports.

Sustained pulmonary vasoconstriction and excessive pulmonary arterial medial hypertrophy significantly contribute to the elevated pulmonary vascular resistance and pulmonary arterial pressure in patients with idiopathic pulmonary arterial hypertension (46). Agonist-mediated PASM contraction and mitogen-mediated cell proliferation play important roles in triggering pulmonary vasoconstriction and stimulating pulmonary vascular remodeling. The agonist- or mitogen-mediated rises in $[Ca^{2+}]_{cyt}$ serve as important stimuli for cell contraction and motility, gene expression, cell cycle progression, and cell growth (6,17,20,21,52). Increased resting $[Ca^{2+}]_{cyt}$ and enhanced Ca^{2+} influx have been demonstrated to cause contraction and stimulate proliferation in PASM, and to increase AP-1 DNA binding activity and stimulate gene expression of growth factors in PAEC (13). Interactions between PASM and PAEC, indeed, have been implicated in the development of pulmonary arterial hypertension (24).

In this study, we examined and compared the histamine-mediated regulation of cytoplasmic $[Ca^{2+}]$ in human PASM and PAEC. Our results indicate that similar pathways of Ca^{2+} influx, such as capacitative Ca^{2+} entry (CCE), can be activated by Ca^{2+} release from internal stores or by depletion of Ca^{2+} from intracellular stores in both cell types. In PASM, the intracellular stores involved in regulating CCE are also the major stores responsible for histamine-mediated Ca^{2+} mobilization. In PAEC, however, the internal stores involved in triggering CCE only partially contribute to histamine-mediated initial transient Ca^{2+} mobilization, but plays a critical role in histamine-mediated oscillatory $[Ca^{2+}]_{cyt}$ changes in the absence of extracellular Ca^{2+} . More importantly, our results indicate that human PAEC may contain a hitherto undescribed histamine-releasable store that is insensitive to CPA, thapsigargin, caffeine, ryanodine, FCCP and bafilomycin, and is minimally present in human PASM.

Methods and Materials

Cell preparation and culture. Human PASM and PAEC from normal subjects (Cambrex, Walkersville, MD) were used in the experiments. PASM were derived from intrapulmonary

arteries of three individuals who were 8 (a male Caucasian), 15 (a male Caucasian, and 26 (a female) years old, respectively, while PAEC were mainly derived from extrapulmonary arteries (including the main pulmonary artery and left and right branches of the main pulmonary artery) from two individuals at age 18 (a male Caucasian) and 24 (a female). Both PASMC and PAEC were cryopreserved at passage 3, replated onto flasks to amplify cell number for 2-3 passages, and then used for the proposed experiments for 2-3 passages. In other words, PASMC and PAEC were used for the experiments at the 5th to 9th passages.

The cells were plated onto cover slips or Petri dishes, and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C in smooth muscle growth medium (SmGM, Cambrex) for PASMC and in endothelium growth medium (EGM, Cambrex) for PAEC. SmGM was composed of smooth muscle basal medium (SmBM) supplemented with 5% fetal bovine serum (FBS), 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 µg/ml insulin. EGM was composed of endothelium basal medium (EBM) supplemented with 2% fetal bovine serum, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 µg/ml insulin. Cells were subcultured or plated onto 25 mm cover slips using trypsin-EDTA buffer when 70-90% confluence was achieved. In some experiments, PAEC were growth-arrested by incubation in EBM for 24 hrs. There was no significant morphological difference between proliferating (cultured in EGM) and growth-arrested (cultured in EBM for 24 hrs) cells. Our previous studies have shown that most of cells (>90%) cultured in the basal medium (without serum and growth factors) are in G₀/G₁ phases of the cell cycle, while more than 66% of cell cultured in the growth medium (with serum and growth factors) are in S/G₂/M phases.

Measurement of [Ca²⁺]_{cyt}. Human PASMC and PAEC on cover slips were placed in a recording cell chamber on the stage of an inverted microscope (Nikon). [Ca²⁺]_{cyt} in single PASMC and PAEC was measured using the Ca²⁺-sensitive fluorescent indicator fluo-4/AM. Cells were loaded with fluo-4 at room temperature for 15 minutes using a physiological salt solution (PSS) containing 10 µM fluo-4/AM, 1.5% DMSO (v/v), and 0.03% cremophor EL (v/v). The fluo-4-loaded cells were then washed with PSS for 15 min to remove excess extracellular dye and allow intracellular esterases to cleave cytosolic fluo-4/AM into active fluo-4. The PSS contained (in mM): 140 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, and 5.6 glucose (pH 7.4 at 22°C). In Ca²⁺-free PSS, 1-2 mM EGTA was added to chelate residual Ca²⁺. In nominally Ca²⁺-free PSS, CaCl₂ was omitted and no EGTA was added.

Excitation (488 nm) was provided by a Xenon arc lamp. Fluorescence emission (>510 nm) was collected using a 40x Nikon Plan Fluor objective (0.75 NA) and a CCD (charge-coupled device) camera (Stanford Photonics, Inc.). "Ca²⁺ images" or fluorescence images based on the fluorescence signals emitted from the cells were acquired at 1 Hz using and stored on a Macintosh computer for later analysis. Although we may refer to the images as "Ca²⁺ images," all the images are simply of Ca²⁺-dependent fluo-4 fluorescence images.

Mn²⁺ quench experiments. Human PASMC and PAEC were incubated in control PSS containing 10 µM fura-2/AM and 1.5% DMSO for 30 min. Cells were then washed for 15 min with PSS to allow cleavage of the loaded fura-2/AM to active fura-2. Excitation of fura-2 to yield Ca²⁺-insensitive signals was selected using a narrow bandwidth excitation filter (360 nm, bandwidth 10 nm). To maximize signal to noise ratio, the excitation was left on for 920 ms and the CCD camera was configured to collect photons throughout this period. Fluorescence emission (510 nm) was collected at 1 Hz. To evaluate Mn²⁺ entry triggered by store depletion, cells were first incubated in Ca²⁺-free media for 5 min and then bathed in Ca²⁺-free media (with 1-2 mM EGTA) containing 10 µM cyclopiazonic acid (CPA) for 15 min. After incubation with CPA, the solution was then switched to nominally Ca²⁺-free solution containing 1 mM Mn²⁺. The narrow bandwidth excitation filter ensured that the signals collected were not

affected by Ca^{2+} ions. The Mn^{2+} quench experiments were designed to be Ca^{2+} -insensitive. The excitation wavelength for this protocol was 360 nm, which is a Ca^{2+} -insensitive wavelength for fura-2. Therefore, fura-2 fluorescence should not change (or should change very little) with increases in $[\text{Ca}^{2+}]_{\text{cyt}}$. Indeed, we did not observe an increase in fluorescence signals (at 360-nm wavelength) in the absence of extracellular Mn^{2+} during application of histamine to cells (both PASM and PAEC), thus validating that the signal we detected with 360-nm excitation was not artificially altered by changes in intracellular Ca^{2+} concentration.

Measurement of the area of cells. The area of PASM and PAEC was determined using the ImageJ software (NIH, ver 1.32j). Briefly, the outlines of the cells were traced manually and the corresponding areas of cells were calculated by the software and recorded in the computer for further analysis. Values of the area were then reported as a normalized value, A/A_0 , where A is the cell area after exposure to histamine and A_0 is the area of the same cell before exposure to histamine. The percentage change of cell area $[(A/A_0)100\%]$ was used to indicate histamine-mediated changes in cell area and size. *Chemicals.* All chemicals were purchased from Sigma (St. Louis, MO) and prepared as stock solutions in the appropriate solvent. Histamine was prepared as an aqueous stock solution and then diluted to the final concentrations using the appropriate saline solution. Cyclopiazonic acid (CPA) and thapsigargin (TG) were dissolved in DMSO to make a stock solution of 10 mM. Aliquots of the stock solution were then diluted into the appropriate solutions on the day of use to their final concentration. Solutions' pH values were measured after addition of the drugs and readjusted to 7.4 when necessary.

A cell-permeable analog of 3,5-bis(trifluoromethyl)pyrazole (BTP), or [N-(4-(3,5-bis((Trifluoromethyl)-1H-pyrazol-1-yl)phenyl)-4methyl-1,2,3-thiadiazole-5-carboxamide] (Calbiochem), that specifically inhibits store-operated Ca^{2+} channels with an IC_{50} approximately 10 nM (23,26,69) was dissolved in DMSO to make a stock solution of 10 mM, which was then diluted to the final concentration in bath solutions on the day of use. A specific inhibitor of phospholipase C (PLC), 1-[6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl] amino] hexyl]-1H-pyrrole-2,5-dione (U-73122; Sigma), was used in some experiments to examine the involvement of PLC in histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$. U-73122 was dissolved in DMSO (50%) and ethanol (50%) to make a stock solution of 25 mM; aliquots were then diluted to the final concentration in bath solutions on the day of use (48,68).

Statistics and data analysis. Regions of interest (ROI) were chosen in each cell to extract fluorescence values. The fluorescence data (F) were then normalized relative to the basal fluorescence value (F_0) of each individual ROI (region of interest) to yield the normalized value F/F_0 . Results are summarized as mean $\pm$ SE of n cells, obtained from multiple cover slips. Each experiment was performed at least 3 times. Statistical significance was tested using paired and unpaired Student's t tests as well as one-way ANOVA with a P value <0.05 taken as significant.

Results

Histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ are comparable in human PASM and PAEC in the absence of extracellular Ca^{2+}

Extracellular application of 12.5 μM histamine (Hist) did not change the cell shape and size (Fig. 1A and B) but significantly increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in a similar fashion in PASM and PAEC bathed in Ca^{2+} -free solution (Fig. 1C), although these two cell types are morphologically different (Fig. 1A). The histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ were composed of an initial rapid transient rise followed by either a sustained or oscillatory increase, due to Ca^{2+} release and Ca^{2+} release-resequestration cycling (Fig. 1C). The oscillatory patterns of histamine-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses were composed of traveling waves that were not always evident at a 1-Hz sampling rate.

The amplitude of histamine-induced initial $[Ca^{2+}]_{cyt}$ transients, although varied individually among cells, was comparable between PASMC and PAEC (Fig. 1C and D). However, in cells that showed $[Ca^{2+}]_{cyt}$ oscillations (or oscillatory responses to histamine), the frequency of histamine-induced oscillations was quite different between PASMC and PAEC; the oscillation with higher frequency was more common in PAEC. As shown in Figure 1E, the oscillations whose frequency is higher than 0.08 Hz were only observed in PAEC; mean frequency in PASMC was 0.04 ± 0.003 Hz (17 out of 35 cells) and in PAEC was 0.06 ± 0.004 Hz (44 out of 111 cells). These observations indicate that histamine-mediated Ca^{2+} release may share the same mechanisms (or Ca^{2+} release channels) in PASMC and PAEC, whereas the mechanisms involved in histamine-mediated $[Ca^{2+}]_{cyt}$ oscillations, which is often closely related to the balance of Ca^{2+} release from and sequestration into various intracellular Ca^{2+} stores (e.g., the sarcoplasmic reticulum, SR), may be different.

In vascular smooth muscle cells, there are at least two functionally distinct receptor-mediated Ca^{2+} pools in the SR: *a*) an IP_3 -releasable store that is sensitive to CPA and thapsigargin (TG), and *b*) a ryanodine-sensitive store that is sensitive to caffeine (8,14,58). The next set of experiments was designed to define *i*) whether the Ca^{2+} -storing capacity, or the concentration of releasable Ca^{2+} , in the SR or endoplasmic reticulum (ER) ($[Ca^{2+}]_{SR/ER}$) is different between PASMC and PAEC; *ii*) which intracellular stores, i.e., the CPA- or caffeine-sensitive stores, are responsible for histamine-mediated $[Ca^{2+}]_{cyt}$ increases in PASMC and PAEC; and *iii*) whether histamine-mediated Ca^{2+} release is derived from different internal stores in PASMC and PAEC.

Passive depletion of intracellularly stored Ca^{2+} with CPA causes capacitative Ca^{2+} entry in PASMC and PAEC

Treatment of human PASMC and PAEC with 10 μ M CPA, an SR/ER Ca^{2+} pump (SERCA) inhibitor (49) that passively depletes intracellularly-stored Ca^{2+} (potentially in the CPA-sensitive or IP_3 -sensitive SR/ER), induced a relatively slow increase in $[Ca^{2+}]_{cyt}$ in the absence of extracellular Ca^{2+} (Fig. 2A). Prolonged or long-term blockade of SERCA with CPA in the absence of extracellular Ca^{2+} , by promoting Ca^{2+} leakage (from the SR/ER to the cytosol) and by inhibiting Ca^{2+} sequestration (from the cytosol to the SR/ER), would deplete Ca^{2+} from the SR/ER (or IP_3 -sensitive stores). Emptying or reduction of $[Ca^{2+}]$ from the SR/ER can then activate store-operated Ca^{2+} channels (SOC) and induce capacitative Ca^{2+} entry (CCE) (41-43). Indeed, after 10-15 min treatment of PASMC and PAEC with 10 μ M CPA in Ca^{2+} -free solution, restoration or reintroduction of extracellular Ca^{2+} induced a rise in $[Ca^{2+}]_{cyt}$ due apparently to CCE (Fig. 2A and B). The results clearly indicate that CCE can be successfully induced in both cell types by a 15-min incubation with CPA.

A set of Mn^{2+} quench experiments (by measuring fura-2 fluorescence at 360-nm wavelength) was conducted to measure the rates of Mn^{2+} influx induced by treatment of the two cell types with CPA. As shown in Figure 2B, no significant differences were observed in CPA-triggered quenching rates between PASMC and PAEC. Blockade of SOC with 1 μ M BTP2 for 15 min abolished the CPA-mediated CCE (Fig. 2C).

The initial rise in $[Ca^{2+}]_{cyt}$ induced by CPA (due to Ca^{2+} leakage from the SR/ER to the cytosol) in the absence of extracellular Ca^{2+} was kinetically different ($P < 0.05$) between PASMC and PAEC (Fig. 2A*b*, left panel), whereas the $[Ca^{2+}]_{cyt}$ increase due to store depletion-mediated CCE was virtually similar in PASMC and PAEC (Fig. 2A*b*, right panel, B and C). The quenching experiments (Fig. 2B) further indicate that CPA-mediated CCE is not different between PASMC and PAEC.

As shown in a separate set of Mn^{2+} quench experiments, the CPA-mediated Ca^{2+} influx was partially inhibited by nifedipine (10 μ M) in PASMC (by 39%; Fig. 3A), but not in PAEC (Fig.

3B). In PASM, CCE may shift the resting membrane potential to a less negative level and activate nifedipine-sensitive voltage-dependent Ca^{2+} channels contributing to the sustained Ca^{2+} influx. However, in PAEC, nifedipine had no effect on the CPA-mediated CCE because these cells do not express voltage-dependent Ca^{2+} channels.

These data indicate that *a*) both PASM and PAEC possess similar mechanisms for triggering CCE when the SR/ER are passively depleted by CPA; *b*) passive “depletion” of Ca^{2+} from the CPA-sensitive SR/ER causes CCE with similar spatiotemporal patterns (e.g., in terms of amplitude and kinetics) in PASM and PAEC; and *c*) the intracellular pools that are responsible for storing Ca^{2+} and releasing Ca^{2+} in response to agonist treatment may be different between PASM and PAEC.

Differential contribution of CPA-sensitive stores to histamine-mediated Ca^{2+} release in PASM and PAEC

Treatment of PASM and PAEC with CPA (10 μM for 15 min) depletes intracellularly stored Ca^{2+} in the CPA-sensitive stores (e.g., IP_3 -sensitive SR/ER). In PASM bathed in Ca^{2+} -free solution, passive depletion of Ca^{2+} from the CPA-sensitive stores, in addition to causing CCE (see Figs. 2 and 3), almost abolished histamine-mediated transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 4A, left panels, and B), whereas in PAEC, CPA treatment did not eliminate histamine-induced rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 4A, right panels, and B). Only a small proportion of PASM showed brief, low amplitude, transient responses to application of histamine after depletion of intracellular stores by CPA, indicating that internal stores in PASM were largely depleted by CPA. However, all of PAEC (51 out of 51 cells examined) treated with CPA still showed a robust transient Ca^{2+} release in response to application of histamine. Maximal mean fluorescence observed was 1.68 ± 0.03 ($n=51$ cells) in PAEC treated with CPA (Fig. 4B). These observations indicate that 15-min CPA treatment could largely deplete intracellular stores that are responsible for histamine-induced Ca^{2+} release in PASM, but could not deplete the histamine-releasable intracellular stores in PAEC. These data further suggest that, in addition to inducing Ca^{2+} influx through Ca^{2+} -permeable channels in the plasma membrane, histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ occur by promoting Ca^{2+} release from different intracellular stores in PASM and PAEC.

CPA treatment altered the kinetics of histamine-mediated Ca^{2+} release in PAEC

As described above, incubation of PAEC with 10 μM CPA, although capable of inducing CCE, failed to eliminate histamine-induced rise in $[\text{Ca}^{2+}]_{\text{cyt}}$. The histamine-induced transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in PAEC pretreated with CPA was, however, lower in amplitude and did not have a sustained component relative to the response observed under control conditions (Fig. 4D). Oscillatory signals in response to histamine could no longer be observed after 15-min incubation of PAEC with CPA. These results suggest that *a*) activation of SOC or induction of CCE in PAEC does not require depletion of all internal Ca^{2+} stores; *b*) CPA-insensitive stores in PAEC do not significantly contribute to store depletion-mediated CCE; *c*) depletion of CPA-sensitive stores in PAEC, although unable to eliminate histamine-induced transient Ca^{2+} release, is sufficient to induce CCE; and *d*) the agonist-releasable pools responsible for storing Ca^{2+} may be different between PASM and PAEC.

Caffeine-sensitive stores are not histamine-releasable stores in PASM and PAEC

In human PASM, short-term (<5 min) treatment with 1 mM caffeine did not induce an obvious increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and had no effect on histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of extracellular Ca^{2+} . Long-term (15 min) pretreatment with caffeine slightly (but insignificantly) increased the histamine-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ release (Fig. 5A). The amplitude of histamine-induced initial peak $[\text{Ca}^{2+}]_{\text{cyt}}$ transient in PASM treated with 1 mM caffeine for 15 min (F/F_0 , 3.22 ± 0.22 , $n=14$ cells from 6 experiments) was slightly, but statistically

significantly ($P < 0.05$), higher than in control PASMC (F/F_0 , 2.48 ± 0.08 , $n = 35$ cells from 4 experiments) and PASMC treated with 1 mM caffeine for 5 min (F/F_0 , 2.51 ± 0.16 , $n = 23$ cells from 5 experiments) (Fig. 5B). The percentage of cells showing oscillatory responses to histamine was fairly similar in control (29%) and caffeine-treated (25% at 5 min and 50% at 15 min) cells. The percentage of cells showing oscillations from previous experiments in Ca^{2+} -free solution was 49% (see Fig. 1C), which was similar to that in cells treated with caffeine for 15 min (50%). These results indicate that most of Ca^{2+} in human PASMC is stored in the CPA-sensitive stores, while very little in “caffeine-sensitive stores.” The Ca^{2+} released from caffeine-sensitive stores can actually be sequestered by CPA-sensitive SERCA to the CPA-sensitive stores in vascular smooth muscle cells (14-16,58); this is probably why histamine-mediated rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca^{2+} release from intracellular stores is slightly enhanced in PASMC treated with caffeine (Fig. 5A, right panel, and B).

Pretreatment of PAEC with 1 mM caffeine in a Ca^{2+} -free solution (with 2 mM EGTA) neither induced a measurable change in $[\text{Ca}^{2+}]_{\text{cyt}}$, nor affected histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 6). In addition, pretreatment of PAEC with 1 μM thapsigargin (TG, for 15 min), similar to treatment with CPA, also failed to abolish histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ when cells were bathed in Ca^{2+} -free solutions (Fig. 7). Actually, the TG-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (compare Fig. 7B left panel with Fig. 2Ab left panel, open circles) and histamine-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ after TG treatment (compare Fig. 7B right panel with Fig. 4B, open circles) were both kinetically comparable to the CPA-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and histamine-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ after CPA treatment. These results indicate that CPA and TG, both inhibitors of SERCA, deplete the same intracellular stores in human PAEC.

Concurrent treatment of PAEC with 10 μM CPA and 1 mM caffeine also had negligible effect on histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca^{2+} release (Fig. 8). The results suggest that caffeine-sensitive stores in PAEC do not significantly contribute to histamine-stimulated Ca^{2+} release. In human PAEC, Ca^{2+} appears to be stored or distributed in various intracellular stores. In addition to the CPA-sensitive store, a CPA- and caffeine-insensitive store exists in human PAEC and plays an important role in mediating histamine-mediated rise in $[\text{Ca}^{2+}]_{\text{cyt}}$.

Mitochondria and bafilomycin-sensitive stores do not contribute to histamine-mediated Ca^{2+} release in PAEC

An agonist-releasable internal store that is insensitive to CPA or caffeine has not been previously described for human PAEC. We therefore sought to define the potential source of the histamine-mediated Ca^{2+} release in PAEC. As shown in Figure 8, treatment of PAEC with 10 μM CPA for a longer time (30 min) was unable to abolish histamine-mediated transient $[\text{Ca}^{2+}]_{\text{cyt}}$ rise (Fig. 8Aa-b and Ba-b) probably because CPA was not able to deplete the histamine-releasable internal stores. CPA has been demonstrated to effectively inhibit SERCA at nanomolar concentrations (49), the inability of prolonged exposure to micromolar levels of CPA to abolish histamine-mediated Ca^{2+} release indicates that histamine-releasable internal stores may not express high levels of CPA-sensitive SERCA.

To examine whether mitochondria and lysosomes are potential histamine-releasable Ca^{2+} stores in PAEC, we investigated the effect of histamine on cells treated with FCCP, a mitochondrial uncoupler that depletes Ca^{2+} from mitochondria (12), and bafilomycin, a vacuolar proton pump inhibitor (or vacuolar H^+ ATPase) that depletes Ca^{2+} from lysosome-related organelles in various cell types (29). Bath application of 5 μM FCCP to PAEC that had been previously incubated in 10 μM CPA (15 min) evoked a small detectable increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (data not shown). A robust brief transient could still be evoked by histamine in PAEC even after treatment (~ 9 min) with combined CPA and FCCP (Fig. 8Ac and Bc). The amplitudes of histamine-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in PAEC treated with CPA (30 min), CPA

+caffeine, and CPA+FCCP were all comparable (Fig. 8Ba-c). Furthermore, combined treatment with CPA+bafilomycin (0.5 μ M for 15 min) also failed to eliminate histamine-induced $[Ca^{2+}]_{cyt}$ transients in PAEC (Fig. 8Ad and Bd). These results suggest that the histamine-induced transient Ca^{2+} release is neither from CPA- and caffeine-sensitive SR/ER, nor from mitochondria or lysosome-like organelles.

To further confirm that ryanodine-sensitive store is not involved in the residual response to histamine, we examined whether histamine was able to induce Ca^{2+} release from intracellular stores in PAEC treated with combined CPA, caffeine and ryanodine. As shown in Figure 9A, histamine still caused a significant increase in $[Ca^{2+}]_{cyt}$ in PAEC treated for 15 min with CPA (10 μ M), caffeine (1 mM) and ryanodine (100 μ M) in the absence of extracellular Ca^{2+} . Since high concentration of ryanodine efficiently blocks Ca^{2+} release through ryanodine receptors, these experiments provide further evidence that there is a unique histamine-releasable store in human PAEC, which is neither the CPA- or TG-sensitive store nor the ryanodine- or caffeine-sensitive store. Although this unique store was insensitive to CPA, TG, caffeine, ryanodine, FCCP and bafilomycin, our study indicated that the histamine-induced Ca^{2+} release from this store still depended on activation of phospholipase C. Inhibition of phospholipase C with U-73122 (50 μ M) in the presence of CPA (10 μ M) abolished the histamine-mediated Ca^{2+} release in PAEC (Fig. 9B). These data suggest that the histamine-releasable (CPA- and TG-insensitive) store is functionally coupled to phospholipase C; receptor-mediated activation of phospholipase C is required to trigger Ca^{2+} release from this store.

Histamine-mediated Ca^{2+} release is not different between proliferating and growth-arrested PAEC

Expression and function of membrane receptors and ion channels responsible for agonist-mediated Ca^{2+} influx and release may change significantly between proliferating and quiescent (or growth-arrested) phenotypes. The experiments described above were all performed in proliferating PAEC cultured in the endothelium growth medium (EGM) containing serum and growth factors. To examine whether PAEC behave differently between proliferating and growth-arrested phenotypes, we examined the effect of histamine on $[Ca^{2+}]_{cyt}$ or Ca^{2+} mobilization in PAEC preincubated for 24 hrs in the endothelium basal medium (EBM) that did not include serum and growth factors. As shown in new Figure 10, histamine-mediated increase in $[Ca^{2+}]_{cyt}$ occurred in 96% of growth-arrested PAEC after treatment with CPA (66 out of 69 cells) (Fig. 10), which was similar to proliferating PAEC (51 out of 51 cells) (Fig. 4). In addition to the percentage of responsive cells out of total cell population examined, the kinetics of histamine-induced $[Ca^{2+}]_{cyt}$ rises or Ca^{2+} released in CPA-treated cell was also comparable between growth-arrested (Fig. 10B right panel) and proliferating (Fig. 8Ba) PAEC. These results indicate that the histamine-releasable and CPA-insensitive intracellular store is functionally present in both quiescent and proliferating PAEC phenotypes.

Discussion

Histamine is a physiologically relevant mediator that has been implicated in allergic, inflammatory, immune responses (2,53,59), as well as in neurotransmission and cell differentiation and maturation. In pulmonary arteries taken from rabbit, guinea pig, and rat, histamine is known to exert vasoconstrictive effects (31,34,50). As seen in studies using pulmonary arteries taken from Wistar-Kyoto and spontaneous hypertensive rats, histamine can also exert endothelium-dependent and -independent relaxation (31,57). In the lung, histamine is synthesized and released mainly from mast cells that are localized to the adventitia of pulmonary vessels. Histamine can affect multiple cell types that carry one or more specific subtypes of G protein-coupled receptors (H receptors) on the plasma membrane. There are four subtypes of surface receptors that mediate responses to histamine (H_1 , H_2 , H_3 , and H_4 receptors). Stimulation with histamine is known to increase hydrolysis of phosphoinositol,

enhance accumulation of inositol phosphates, and elevate $[Ca^{2+}]_{cyt}$ (22). In the human pulmonary vasculature, histamine exerts dual functional effects, both vasoconstrictive and vasodilatory effects, mediated by receptors located on both PASMC and PAEC (4). The contractile response of pulmonary vessels to histamine is mediated by H_1 -receptors on PASMC, and the relaxant response is mediated by both H_2 receptors on PASMC and H_1 -receptors in PAEC (9,31,39,50). The endothelial production of nitric oxide and prostacyclin upon activation of H_1 receptors on PAEC is involved in histamine-mediated pulmonary vasodilation (30,31,39,55). Furthermore, histamine-mediated Ca^{2+} mobilization from intracellular stores evokes oscillatory K^+ and Cl^- currents by opening Ca^{2+} -activated K^+ channels and Ca^{2+} -activated Cl^- channels (64) that are highly expressed in PASMC and PAEC.

CPA-sensitive Ca^{2+} stores contribute to histamine-mediated Ca^{2+} release

Upon activation of respective receptors by agonists or ligands (e.g., histamine), Ca^{2+} release may be derived from one or more types of intracellular Ca^{2+} stores including the SR/ER, acidic stores, Golgi apparatus, and mitochondria. Ca^{2+} can be released via action of IP_3 on IP_3 receptors (IP_3R) located on the internal store membrane and/or via Ca^{2+} -induced Ca^{2+} release (CICR) from stores that contain ryanodine receptors. The SR/ER Ca^{2+} stores can be further subdivided into spatially and functionally distinct sub-compartments that can unload and refill separately from each other. CPA- and IP_3 -as well as caffeine- and ryanodine-sensitive stores have been well demonstrated in vascular smooth muscle cells, neurons, and astrocytes (14-16,58).

Our results from the current study indicate that the histamine-mediated Ca^{2+} release in human PASMC is derived mainly from CPA- and IP_3 -sensitive stores because incubation of cells in 10 μM CPA was able to eliminate the response to stimulation with histamine. In PAEC, a major proportion of Ca^{2+} is also released from CPA- and IP_3 -sensitive stores in response to histamine stimulation, since treatment of PAEC with CPA significantly attenuated the histamine-mediated rise in $[Ca^{2+}]_{cyt}$ in the absence of extracellular Ca^{2+} . In addition, pre-incubation of PAEC with CPA also altered the kinetics of the histamine-mediated rise in $[Ca^{2+}]_{cyt}$; the CPA treatment eliminated histamine-induced $[Ca^{2+}]_{cyt}$ oscillations. These observations suggest that CPA- and IP_3 -sensitive stores play a significant role in histamine-stimulated increases in $[Ca^{2+}]_{cyt}$ in both PASMC and PAEC.

Another significant difference between PASMC and PAEC shown in our study was that histamine-mediated oscillation frequency was greater in PAEC than in PASMC (Fig. 1E). Ca^{2+} oscillations are regulated by: *a*) the balance between Ca^{2+} release from intracellular stores through Ca^{2+} release channels and Ca^{2+} sequestration back into the stores via Ca^{2+} - Mg^{2+} ATPase in the SR/ER (SERCA), *b*) the balance between Ca^{2+} influx through Ca^{2+} channels and Ca^{2+} extrusion via Ca^{2+} - Mg^{2+} ATPase in the plasma membrane, and *c*) the subcellular organization of the Ca^{2+} release and sequestration system (18,65). Since PAEC are morphologically smaller than PASMC, and tend to have a much smaller cytosol area or store-to-surface distance than PASMC, the relatively higher frequency of Ca^{2+} oscillations in PAEC might be due to *i*) a shorter distance between the SR/ER (or Ca^{2+} release channels and SERCA) and the plasma membrane (or Ca^{2+} -permeable channels and Ca^{2+} pumps), *ii*) a smaller subplasmalemmal area, and *iii*) different organization of Ca^{2+} release and uptake systems. It is unclear whether activation threshold of Ca^{2+} channels and Ca^{2+} pumps in the SR and plasma membrane differs between human PASMC and PAEC.

Depletion of CPA-sensitive Ca^{2+} stores is sufficient to induce CCE in PASM and PAEC

Maintaining a high concentration (or capacity) of Ca^{2+} in the SR/ER ($[\text{Ca}^{2+}]_{\text{SR/ER}}$) is not only important in the signal transduction process required for cell contraction, migration, and gene expression, but also necessary for appropriate lipid and protein synthesis in the SR/ER. Depletion of Ca^{2+} from intracellular stores (e.g., the SR/ER) as a result of activation of IP_3 receptors or inhibition of SERCA has been demonstrated to mediate CCE by opening SOC in many cell types. CCE plays a significant role in maintaining the filling state of SR/ER (41). The intracellular Ca^{2+} store that contributes to regulating SOC activity or controlling CCE can be depleted actively by opening Ca^{2+} release channels or passively by blocking the Ca^{2+} - Mg^{2+} ATPase in the SR/ER. Our results from this study indicate that emptying or reduction of IP_3 -sensitive stores by CPA is sufficient to induce CCE in both human PASM and PAEC. The CPA-mediated CCE in PASM and PAEC is sensitive to the SOC blocker, BTP2 (23, 26,69). At present, the exact mechanisms involved in the induction of CCE are still unclear; several models have been described: *a*) chemical coupling mechanism in which a soluble messenger released from intracellular stores serves as a trigger to open plasmalemmal SOC and induce CCE; *b*) conformational coupling mechanism in which the direct or indirect (via cytoskeleton) interaction between SOC in the plasma membrane and IP_3 receptors in the SR mediates opening of SOC and induction of CCE; and *c*) physical coupling mechanism in which the SR is physically, yet indirectly, tethered to the sarcolemmal SOC by one or more scaffolding proteins (40,42,45,61).

Histamine-mediated Ca^{2+} release derives from multiple internal stores in PAEC

A significant difference exists between PASM and PAEC with regards to the stores involved in the response to stimulation with histamine. In PASM, pretreatment with CPA, which depletes CPA- or IP_3 -sensitive stores, almost abolished histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of extracellular Ca^{2+} . In PAEC, pretreatment with CPA did not eliminate histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca^{2+} mobilization, although CPA treatment affected the kinetics of histamine-mediated Ca^{2+} release. These data indicate that the CPA-sensitive Ca^{2+} stores are not the only stores that are responsible for histamine-mediated Ca^{2+} release in PAEC, although the level of Ca^{2+} in these stores contributes to the regulation of CCE and activity of SOC.

In addition to the SR/ER, the membrane of other cytoplasmic organelles, such as the mitochondrion, Golgi apparatus, lysosome, peroxisome, and endosome, also contain transport proteins that are responsible for the import and export of specific metabolites and ions (e.g., Ca^{2+} , Na^+ , and K^+). In eukaryotic cells, approximately half the total area of membrane encloses the SR/ER, while about 15% of the total cell volume is occupied by the SR/ER. Therefore, the SR/ER is undoubtedly the most predominant intracellular Ca^{2+} store in mammalian and human cells. Nevertheless, in many cell types, mitochondria, Golgi apparatus, lysosome, and endosome have also been shown to function as intracellular Ca^{2+} stores (36). Since the CPA-sensitive SR/ER is not the only intracellular organelle to store agonist-releasable Ca^{2+} in PAEC, it would be important to define other potential stores that are responsible for histamine-mediated Ca^{2+} release.

Pretreatment of PAEC with the combination of *a*) CPA (10 μM), caffeine (1 mM) and ryanodine (100 μM); *b*) CPA and FCCP (5 μM); or *c*) CPA and bafilomycin A1 (0.5 μM) (10) all failed to eliminate histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of extracellular Ca^{2+} . In PASM bathed in Ca^{2+} -free solutions, however, pretreatment with effective doses of CPA

(5-10 μM) alone almost abolished histamine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. These results suggest that PAEC contain an alternate source of histamine-releasable Ca^{2+} that is minimally present in PASMC. This internal Ca^{2+} store is insensitive to CPA (or TG) and caffeine (or ryanodine), so it is not the CPA- and IP_3 -sensitive SR/ER or caffeine- and ryanodine-sensitive SR/ER.

An intimate relationship exists between the SR/ER and mitochondria. Ca^{2+} released from the SR/ER can be taken up by neighboring mitochondria and result in a transient increase in mitochondrial $[\text{Ca}^{2+}]$. The continuous flux of Ca^{2+} into and out of mitochondria, which occurs during stimulation with histamine of some cell types, is then linked to the refilling of nearby SR/ER stores (3,33,35). The inability of CPA and FCCP (a proton ionophore that dissipates the mitochondrial membrane potential and depletes Ca^{2+} from mitochondria) to eliminate histamine-mediated Ca^{2+} release, however, suggests that mitochondria do not directly contribute to the histamine-induced elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC.

The Ca^{2+} pools (or the SR/ER) that are resistant to SERCA inhibitors (e.g., CPA, thapsigargin) have been shown in some mammalian cells (56,63). The bafilomycin-sensitive V-type ATPases (10) that are associated with acidic Ca^{2+} storage compartments may also exist in the SR/ER and other unknown internal stores. However, the inability of CPA and bafilomycin to eliminate histamine-mediated Ca^{2+} mobilization indicates that the internal stores utilizing bafilomycin-sensitive V-type ATPases do not directly contribute to histamine-mediated Ca^{2+} release in human PAEC.

Taken together, the results from this study indicate that human PAEC possess a unique internal Ca^{2+} store that is minimally present in PASMC. The identity of this PAEC-specific internal Ca^{2+} store is presently unknown; whether it is the Golgi apparatus requires further investigation. Identification of this novel Ca^{2+} pool in human PAEC, and its possible contribution to normal or pathologic cellular processes represents a new dimension in our understanding of Ca^{2+} homeostasis and its effects on pulmonary vascular endothelial function. Although it seems not to be coupled to capacitative Ca^{2+} entry, this unique internal Ca^{2+} store (insensitive to CPA, TG, caffeine, ryanodine, FCCP and bafilomycin) has sufficient capacity to store and release Ca^{2+} to mediate histamine-induced functional effect on PAEC. This unique store may serve as a “reserved” resource for agonist- and receptor-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$, especially when PAEC are exposed to multiple agonists and growth factors. Amplitude and frequency modulation of intracellular Ca^{2+} signals play important roles in regulating cell contraction, mobility, proliferation, differentiation, and apoptosis, this unique Ca^{2+} store in human PAEC may greatly contribute to the agonist- (or histamine-) mediated activation of cytoplasmic enzymes, such as nitric oxide synthase, adenylyl cyclases and calmodulin kinases (CaMK), and of transcription factors, such as c-Jun, c-Fos, and CREB (5,7,54).

Summary

A rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC serves as a major trigger for pulmonary vasoconstriction, whereas an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC, in addition to inducing cell mobility and migration, may also be an important stimulus for activating enzymes that produce endothelium-derived relaxing factors (e.g., nitric oxide). Our data from this study demonstrate that *a*) PASMC and PAEC share the same intracellular stores and mechanisms for triggering CCE, but use different intracellular stores and mechanisms for agonist (e.g., histamine)-mediated Ca^{2+} mobilization; *b*) both PASMC and PAEC are able to generate oscillations in the absence of extracellular Ca^{2+} , the Ca^{2+} oscillation in PASMC seems to be mainly due to release-uptake cycling through the same store (i.e., CPA-sensitive store), while the Ca^{2+} oscillation in PAEC is predominantly caused by release-uptake cycling among different stores; *c*) there are multiple intracellular

Ca²⁺ stores in PAEC that ensure agonist-mediated Ca²⁺ release or signaling, a unique internal store that is not required for triggering CCE but partially involved in agonist-induced Ca²⁺ release exists in PAEC; and *d*) the major intracellular stores in PASMC are CPA-sensitive stores that are not only responsible for agonist-mediated Ca²⁺ mobilization but also contribute to controlling the activity of SOC and regulation of CCE.

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Fig. 1

. Characteristics of histamine-mediated Ca^{2+} release in human PASMC and PAEC. **A:** The contrasting morphology typical of human PASMC and PAEC in which $[\text{Ca}^{2+}]_{\text{cyt}}$ was measured before (control) and during (Hist) application of histamine ($12.5 \mu\text{M}$). The left column shows PASMC and PAEC under basal conditions while the right column shows the same cells during stimulation with histamine. **B:** Summary data (means $\pm$ SE) showing the percentage changes in area of PASMC ($n=27$ cells) and PAEC ($n=42$ cells) after application of histamine. Cell area was determined from images like in **A**. Values are reported as the normalized value A/A_0 , where A is area during exposure to histamine, A_0 is cell area in basal condition. **C:** Representative records of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes (denoted by F/F_0) in PASMC (left panels) and PAEC (right panels) before, during, and after application of $12.5 \mu\text{M}$ histamine in Ca^{2+} -free (0Ca , with 2 mM EGTA) solution. Each trace in **C** represents F/F_0 changes in an area of interest (AOI) located in the cytosol of a single cell. **D:** Histogram showing amplitude distributions of the histamine-mediated initial peak $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in PASMC (upper panel) and PAEC (lower panel). Mean fluorescence ratios (F/F_0) are comparable between PASMC (2.48 ± 0.08 , $n=35$ cells) and PAEC (2.48 ± 0.04 , $n=111$ cells). **E:** Histogram showing frequency distribution of the histamine-induced oscillatory changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC (upper panel) and PAEC (lower panel). 48% of PASMC (17/35) and 40% of PAEC (44/111) exhibited oscillatory changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ upon stimulation with histamine.

Fig. 2

. CPA-mediated passive store depletion induces capacitative Ca^{2+} entry (CCE) in human PASMC and PAEC. A: Representative records (a) of $[Ca^{2+}]_{cyt}$ changes (denoted by F/F_0) in PASMC (left panels) and PAEC (right panels) before, during, and after extracellular application of 10 μM CPA in the absence (0Ca) or presence of extracellular Ca^{2+} . Summarized data (b, means $\pm$ SE) showing the time course of CPA-mediated changes in $[Ca^{2+}]_{cyt}$ in PASMC (closed circles, $n=36$ cells) and PAEC (open circles, $n=23$ cells). Reintroduction of extracellular Ca^{2+} approximately 15 min after CPA treatment increases $[Ca^{2+}]_{cyt}$ apparently due to CCE (right panels). The records of CCE-mediated $[Ca^{2+}]_{cyt}$ changes in the absence of extracellular Ca^{2+} (left panels in PASMC and PAEC) and the records of CCE-mediated

$[Ca^{2+}]_{cyt}$ changes after restoration of extracellular Ca^{2+} (right panels in PASMC and PAEC) are from different cells. *B*: Representative records (*a*) and summarized data (*b*, means $\pm$ SE) showing Mn^{2+} quench of fura-2 fluorescence signals at 360 nm in PASMC (closed circles, n=24 cells) and PAEC (open circles, n=16). The cells were pre-treated with CPA (10 μ M) for 15 min before addition of Mn^{2+} . *C*: Summarized data (means $\pm$ SE) showing the time course of CCE-induced increases in $[Ca^{2+}]_{cyt}$ (denoted by fluo-4 fluorescence signals, F/F_0) in PASMC (*a*, n=43) and PAEC (*b*, n=59) in the absence (Cont) or presence (BTP2) of 1 μ M BTP2. The control curves (open circles) were constructed from the data presented in *Ab* (right panel).

Fig. 3

. Effect of nifedipine on CPA-mediated Mn^{2+} entry in human PASMC and PAEC. The left panels of A and B represent the background photobleaching rate (in the absence of extracellular Mn^{2+}) associated with the experiments for each cell type (PASMC and PAEC). Right panels: Summarized data (means $\pm$ SE) showing Mn^{2+} quench of fura-2 fluorescence signals in PASMC (A) and PAEC (B) in the absence (CPA; n=25 for PASMC and n=59 for PAEC) or presence (CPA+Nif; n=40 for PASMC and N=137 for PAEC) of 1 μ M nifedipine. The cells were pretreated with 10 μ M CPA for 15 min before addition of Mn^{2+} . The gray lines in right panels denote extrapolation of the data points (0-35 sec) before extracellular Mn^{2+} was applied to cells (the time for Mn^{2+} application is indicated by the vertical broken lines).

Fig. 4

. Effects of pretreatment with CPA on histamine-mediated Ca^{2+} release in human PASMC and PAEC. **A**: Representative traces of histamine-stimulated changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ (denoted by F/F_0) in PASMC (left panels) and PAEC (right panels) after incubation in Ca^{2+} -free (0Ca) solution containing 10 μM CPA. A small proportion of PASMC demonstrated low amplitude and transient responses, while a majority of the cells showed no response to histamine (Hist). All PAEC, in contrast, showed a robust transient response to stimulation with histamine. **B** and **C**: Summarized data (means $\pm$ SE) showing the time course (**B**) and averaged maximal values (**C**) of histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ (F/F_0) in PASMC (closed circles and solid bar, $n=36$) and PAEC (open circles and open bar, $n=51$) after treatment with 10 μM CPA in 0Ca solution. *** $P<0.001$ vs. PASMC. **D**: Comparison of the amplitude (left panel) and kinetics (right panel) of histamine-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ (denoted by F/F_0) in PAEC treated with (CPA, $n=111$) or without (Cont; the curve was constructed from the data shown

in Fig. 1B) 10 μ M CPA. Data shown in the right panel are normalized to the maximal level of changes in $[Ca^{2+}]_{cyt}$; the maximum F/F_0 value is set at 100% according to the formula: $[(F/F_0)-1]/(F_{max}-1)$. The histamine-mediated increases in $[Ca^{2+}]_{cyt}$ are lower in amplitude and faster in decay after treatment with CPA.

Fig. 5

. Effect of pretreatment with caffeine on histamine-mediated Ca^{2+} release in human PASMC. **A:** Summarized data (means $\pm$ SE) showing the time course of $[Ca^{2+}]_{cyt}$ changes in response to extracellular application of 1 mM caffeine (Caf, in Ca^{2+} -free solution; left panel, $n=15$) and to histamine (Hist, right panel) in control cells (open circles) and cells pretreated with 1 mM caffeine (Caf) for 5 (gray triangles) or 15 (closed circles) min ($n=14-24$). **B:** Summarized data (means $\pm$ SE) showing the amplitude of histamine-mediated initial peak increase in $[Ca^{2+}]_{cyt}$ (left panel). * $P<0.05$ vs. open and grey bars. **C:** Percentage of the cells in which oscillatory $[Ca^{2+}]_{cyt}$ increases were observed in response to histamine in control cells and cells treated with caffeine (5 or 15 min).

Fig. 6

. Effect of pretreatment with caffeine on histamine-mediated Ca^{2+} release in human PAEC. Representative traces (A) and summarized data (B, means $\pm$ SE) of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes (denoted by F/F_0) in response to 1 mM caffeine (Caf) in the Ca^{2+} -free (0Ca) solution (left panels, n=93) and to 12.5 μM histamine (Hist) in cells incubated in Caf-containing 0Ca solution (for 15 min, right panels, n=77). Application of caffeine alone did not evoke robust increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ (only 6 out of 93 cells showed very small and brief transients in response to caffeine). Histamine-mediated oscillatory changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ can still be observed in some cells in the presence of caffeine (not shown).

Fig. 7

. Effects of pretreatment with thapsigargin (TG) on histamine-mediated Ca^{2+} mobilization in human PAEC. *A*: Representative traces of $[Ca^{2+}]_{cyt}$ changes in PAEC in response to application of 1 μ M TG (left panels) and to application of 12.5 μ M histamine (after $\sim$ 15 min of exposure to TG) (right panels), under Ca^{2+} -free conditions. *B*: Summarized data (means $\pm$ SE) of $[Ca^{2+}]_{cyt}$ changes in PAEC (denoted by F/F_0) in response to 1 μ M TG in Ca^{2+} -free (0Ca) solution (left panel, n=76) and to 12.5 μ M histamine (Hist) in cells incubated in TG-containing 0Ca solution (for 15 min, right panel, n=80).

Fig. 8

. Effects of pretreatment with CPA, caffeine, FCCP, and bafilomycin on histamine-mediated Ca^{2+} mobilization in human PAEC. Representative traces (A) and summarized data (B, means $\pm$ SE) of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes (denoted by F/F_0) in response to extracellular application of 12.5 μM histamine (Hist) in Ca^{2+} -free (0Ca) solution in PAEC pretreated with 10 μM CPA alone for 30 min (a, $n=107$), CPA and 1 mM caffeine (b, $n=114$), CPA and 5 μM FCCP (c, $n=71$), or CPA and 0.5 μM bafilomycin (d, $n=80$). Pretreatment with CPA alone, CPA+caffeine, CPA+FCCP, or CPA+bafilomycin did not significantly affect histamine-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC superfused with Ca^{2+} -free solution.

Fig. 9

. Effect of pretreatment with the combination of CPA, caffeine and ryanodine, or pretreatment with U-73122, on histamine-mediated Ca^{2+} mobilization in human PAEC. **A:** Representative traces (*a*) and summary data (*b*, means $\pm$ SE) of $[Ca^{2+}]_{cyt}$ changes in PAEC in response to application of CPA (10 μ M), caffeine (Caf, 1 mM) and ryanodine (Ry, 100 μ M) (left panels, $n=38$) and to application of 12.5 μ M histamine (after ~ 15 min of exposure to CPA+Caf+Ry) (right panels, $n=42$), under Ca^{2+} -free conditions. **B:** Representative traces (*a*) and summary data (*b*, means $\pm$ SE) of $[Ca^{2+}]_{cyt}$ changes in PAEC in response to application of histamine (after ~ 15 min of exposure to 10 μ M CPA and 50 μ M U-73122). The data ($n=20$) in *b* show abolition of the response to histamine in PAEC that have been pretreated with CPA+U73122.

Fig. 10

. Effect of pretreatment with CPA on histamine-mediated Ca^{2+} mobilization in growth-arrested human PAEC. Representative traces (A) and summarized data (B, means $\pm$ SE) of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in PAEC (precultured in EBM for 24 hrs) in response to application of $10 \mu\text{M}$ CPA (left panels, $n=74$) and to application of $12.5 \mu\text{M}$ histamine (after ~ 15 minutes of exposure to CPA) (right panels, $n=69$), under Ca^{2+} -free conditions. Almost all (66 out of 69) growth-arrested cells treated with CPA showed a response to bath application of histamine, similar to proliferating PAEC cultured in EGM.