Caveolins and intracellular calcium regulation in human airway smooth muscle

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Praekash, YS, Thompson, MA, Vaa, B, Matabdin, I, Peterson, TE, He, T, Pabelick, CM. Caveolins and intracellular calcium regulation in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 293: L1118–L1126, 2007. First published August 17, 2007; doi:10.1152/ajplung.00136.2007.—Regulation of intracellular Ca2+ concentration ([Ca2+]i) is a key factor in airway smooth muscle (ASM) tone. In vascular smooth muscle, specialized membrane microdomains (caveolae) expressing the scaffolding protein caveolin-1 are thought to facilitate cellular signal transduction. In human ASM cells, we tested the hypothesis that caveolae mediate Ca2+ responses to agonist stimulation. Fluorescence immunocytochemistry with confocal microscopy, as well as Western blot analysis, was used to determine that agonist receptors (M1 muscarinic, bradykinin, and histamine) and store-operated Ca2+ entry (SOCE)-regulatory mechanisms colocalize with caveolin-1. Although caveolin-2 coexpressed with caveolin-1, caveolin-3 was absent. In fura 2-loaded ASM cells, [Ca2+]i responses to 1 μM Ach, 10 μM histamine, and 10 nM bradykinin, as well as SOCE, were attenuated (each to a different extent) after disruption of caveolae by the cholesterol-chelating drug methyl-β-cyclodextrin. Transfection of ASM cells with 50 nM caveolin-1 small interfering RNA (siRNA), significantly disrupts [Ca2+]i expression and blunted [Ca2+]i responses to bradykinin and histamine, as well as SOCE. However, the response to Ach was less intense. These results indicate that caveolae are present in ASM and that caveolin-1 contributes to regulation of [Ca2+]i, responses to agonist.

intracellular signaling pathway; methyl-β-cyclodextrin; small interfering RNA; store-operated calcium entry

There is considerable evidence for flask-shaped plasma membrane invaginations (caveolae) in different cell types, including smooth muscle (8, 14, 21, 44, 47, 53, 54). Rich in cholesterol and sphingolipids, caveolae are known to express any of three caveolin proteins (caveolin-1, caveolin-2, and/or caveolin-3), which are thought to be involved in maintenance and facilitation of cell structure and function (8, 14, 21, 44, 47, 53, 54). Caveolins may act as scaffolding sites to facilitate proximity of different signaling proteins (simply similar to t tubules in striated muscle). One potential role for caveolins may be regulation of intracellular Ca2+ concentration ([Ca2+]i). Indeed, in vascular smooth muscle, which expresses caveolin-1, caveolae have been found to be in close proximity to the sarcoplasmic reticulum (SR), which is a key component of [Ca2+]i regulation (36, 45). In airway smooth muscle (ASM), it has been reported that the plasma membrane fraction enriched in caveolae contains several Ca2+-regulatory proteins (9). Whether caveolins actually regulate ASM [Ca2+]i is not known.

In ASM, elevation of [Ca2+]i by bronchoconstrictors such as ACh, histamine, and bradykinin involves Ca2+ release from SR stores and Ca2+ influx across the plasma membrane (3, 7, 27, 34, 40). Ca2+ influx occurs through voltage-gated (56) and receptor-gated (31) channels. Controlled Ca2+ influx in response to SR Ca2+ depletion also occurs (3, 34), allowing for replenishment of intracellular Ca2+ stores [store-operated Ca2+ entry (SOCE)] (38, 41, 43).

In the present study, we examined the role of caveolin-1 in modulation of [Ca2+]i in human ASM cells. We demonstrate that caveolin-1 is indeed present in the plasma membrane of human ASM and colocalizes with receptors of several bronchoconstrictor agonists. Although caveolin-2 is coexpressed with caveolin-1, we could not detect caveolin-3. Furthermore, we demonstrate that disruption of caveolae by cyclodextrins, as well as inhibition of caveolin-1 expression via small interfering RNA (siRNA), significantly disrupts [Ca2+]i responses to bronchoconstrictors and modulates SOCE. These data highlight the importance of caveolae and caveolin-1 in [Ca2+]i regulation in human ASM.

MATERIALS AND METHODS

Isolation of human ASM cells. Human bronchial smooth muscle cells were obtained from surgical waste lung tissue from patients undergoing thoracic surgery at Mayo Clinic Rochester with approval of the Mayo Clinic Institutional Review Board. No patient information about tissues deemed surgical waste by the Department of Pathology was available to us. Tissues were initially placed in Hanks’ balanced salt solution (HBSS; Invitrogen) with 2.5 mM extracellular Ca2+. Bronchioles were identified and freed of cartilage, epithelium, and surrounding tissues, and ASM cells were isolated using previously described techniques (40). Cells were plated in sterile culture flasks and grown in a humidified incubator 95% air-5% CO2 in balanced salt solution (HBSS; Invitrogen) with 2.5 mM extracellular Ca2+.

Preparation of caveolar membranes. Caveolin-rich membranes were prepared from ASM cells as previously described by Wang et al. (52). Briefly, ASM cells were grown in 150-mm petri dishes until confluent. Cells were harvested by scraping, homogenized in cold buffer A (0.25 M sucrose, 1 mM EDTA, and 20 mM tricine, pH 7.8), layered onto a 30% Percoll gradient, and centrifuged at 84,000 g for 30 min. The plasma membrane fraction was extracted, and its volume was increased to 2 ml with buffer A. The crude membrane fraction was

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then sonicated three times (5 s each), resuspended in a 23% solution of OptiPrep, and transferred to a centrifuge tube. A linear 20%-10% OptiPrep gradient was layered on top of the tube, which was centrifuged at 52,000 g for 90 min. The upper membrane layer (containing caveolae) was collected for further experimentation. Cholesterol depletion of ASM was achieved by incubation with 10 mM methyl-β-cyclodextrin (CD), a cholesterol-binding drug, in serum-free medium for 1 h, and the cells were fractionated by density gradient centrifugation (see above).

Confocal immunofluorescence microscopy. ASM cells were grown on four-chambered Lab-Tek chambers (Nalgene Nunc, Rochester, NY) to 50% confluence and fixed in ice-cold methanol for 15 s. Cells were then washed three times in 0.1 M Tris-buffered saline (TBS), permeabilized with 0.1% Triton X-100 in TBS for 15 s, washed in TBS, and blocked for 60 min in 4% normal donkey serum. Cells were then incubated overnight at 4°C in TBS only (unstained control) or 1 μg/ml of the following antibodies: mouse anti-caveolin-1, mouse anti-caveolin-2, mouse anti-caveolin-3, rabbit anti-M1 muscarinic receptor, rabbit anti-B2 bradykinin receptor, rabbit anti-histamine H1 receptor, rabbit anti-Ca2⁺-activated transient receptor protein channel (TRPC)-type 4 (TRPC4) or anti-TRPC6 [putative SOCE channels previously shown to be expressed in ASM (3)], and mouse anti-stromal interaction molecule type 1 (STIM1; recently shown to be the trigger for SOCE in different cell types including ASM (13, 20, 33, 39, 42, 58)]. Cells were then washed in TBS and incubated for 1 h in Cy5-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-mouse secondary antibodies (1:200 dilution; Jackson ImmunoResearch). Cells incubated with secondary antibodies served only as staining controls. Labeled cells were visualized using a ×100/1.3 NA oil immersion objective lens on an Olympus FluoView laser scanning confocal microscope equipped with Ar and Kr lasers (568- and 647-nm lines). Image acquisition parameters were set on the basis of the fluorescence intensity of unstained and secondary antibody-stained samples. Image registration for two-color imaging was verified using multifluorescent beads. Fluorescence intensities of caveolin isoform and [Ca2⁺]-regulatory protein staining were determined using fixed laser and photomultiplier settings across different samples.

Western blot analysis. Proteins were separated by SDS-PAGE (Criterion Gel System, Bio-Rad, Hercules, CA; 15% or 4–15% gradient gels) and transferred to polyvinylidene difluoride membranes (Bio-Rad) for 60 min. Membranes were blocked for 1 h with 5% milk in TBS containing 0.1% Tween and then incubated overnight at 4°C with anti-caveolin-1, anti-caveolin-2, or anti-caveolin-3 (1:1,000 dilution), antibodies against ACh, bradykinin, or histamine receptors, or antibodies against TRPC4, TRPC6, or STIM1 (1:200 dilution; see Confocal immunofluorescence microscopy). After the membranes were washed three times with Tris-buffered saline + 0.1% Tween, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and signals developed by Supersignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL).

Caveolin-1 knockdown by siRNA. A caveolin-1 siRNA duplex targeting the open reading frame of bovine caveolin-1 mRNA (223–241 bases; 5’-CCA GAA GGA ACA CAC AGU U-dTdT-3’) and a negative control siRNA (5’-GGG CCG UUU GUA GGA UUC G-ddTdT-3’) were selected for caveolin-1 knockdown. siRNA duplex oligonucleotides were purchased from Dharmacon (Lafayette, CO). ASM cells at 60% confluence were transfected using 50 nM siRNA and Lipofectamine 2000 (Invitrogen) in DMEM-Ham’s-F-12 medium without FBS. Fresh growth medium was added 6 h after transfection, and the cells were analyzed for 48 h. The efficacy of siRNA knockdown was verified by Western analysis of decreased caveolin-1 expression.

[Ca2⁺]i imaging. ASM cells were incubated in 5 μM fura 2-AM (Molecular Probes, Eugene, OR) for 60 min at room temperature and visualized with a fluorescence imaging system (MetaFluor, Universal Imaging, Downingtown, PA) on a Nikon Diaphot inverted microscope (Fryer Instruments, Edina, MN). Cells were initially perfused with HBSS [2.5 mM Ca2⁺, room temperature (23°C)], and a baseline fluorescence was established. A custom-built fluid level controller allowed cell perfusion with rapid (<300 ms) exchange of perfusate. [Ca2⁺]i responses of 10 cells per chamber were obtained for individual, software-defined regions of interest. Fura 2-loaded cells were alternately excited at 340 and 380 nm with a Lambda 10-2 filter changer (Sutter Instrument, Novato, CA). Fluorescence emissions were collected separately approximately every 0.75 s for each wavelength with a 510-nm barrier filter. Images were acquired with a Photometric Cascade digital camera system (Roper Scientific, Tucson, AZ), and results were expressed as the ratio of the 340-nm to 380-nm wavelengths. Previously described calibration procedures were used to quantify [Ca2⁺], from fura 2 levels (17, 40). For agonist stimulation, amplitudes of [Ca2⁺]i responses were calculated as the difference between peak Ca2⁺ and baseline Ca2⁺ before stimulation. The subsequent plateau level was measured from the same resting baseline. For SOCE measurements, the amplitude of the cyclopiazonic acid (CPA) response was taken from the resting baseline (in zero Ca2⁺) to the plateau [Ca2⁺], level after CPA stimulation, whereas SOCE was measured relative to this CPA plateau.

Materials. Chemicals and supplies were obtained from Sigma (St. Louis, MO) unless otherwise mentioned. Tissue culture reagents, including DMEM-Ham’s-F-12 medium and FBS, were obtained from Invitrogen (Carlsbad, CA). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise noted: rabbit polyclonal anti-caveolin-1, mouse monoclonal anti-caveolin-1, anti-caveolin-2, and anti-caveolin-3 from BD Transduction Laboratories (Franklin Lakes, NJ); rabbit polyclonal anti-muscarinic M1 ACh receptor, rabbit polyclonal anti-histamine H1 receptor, rabbit polyclonal anti-bradykinin B2 receptor, and rabbit polyclonal anti-TRPC4 and anti-TRPC6 from Alomone Labs (Jerusalem, Israel); and mouse anti-STIM1 from Novus Biologicals.

Statistical analysis. ASM cells were obtained from at least five bronchial samples. All biochemical and molecular biology experiments (e.g., Western analysis and siRNA) were repeated at least three times. [Ca2⁺]i experiments were performed in ≥20 cells each from 5 bronchial samples, although not all protocols were performed on each sample. Drug effects on [Ca2⁺]i were expressed as the ratio of the 340-nm to 380-nm wavelengths. Statistical significance was established at P < 0.05. Values are means ± SE.

RESULTS

Caveolin expression in ASM. Methanol-fixed human ASM cells displayed significant immunostaining of the plasma membrane for caveolin-1, as detected by Cy3 fluorescence imaging (Fig. 1). Interestingly, fixation of ASM cells with 2% paraformaldehyde prevented membrane staining of caveolin-1, although intracellular (e.g., Golgi apparatus) staining persisted. This may reflect a peculiarity of the primary antibody; however, antibodies from at least two vendors produced similar results.

Receptors for ACh, histamine, and bradykinin were detected at the plasma membrane of cells containing caveolin-1 (Fig. 1). Immunostaining localized caveolin-2 to the plasma membrane (similar to caveolin-1). However, staining was generally weaker for caveolin-2 than for caveolin-1 (Fig. 2). In human ASM cells, caveolin-3 was not detected by immunostaining (Figs. 1 and 2). The SOCE-triggering protein STIM1 was also expressed at the plasma membrane, colocalized with caveolin-1. Interestingly, TRPC4 at the plasma membrane colocalized with caveolin-1 to a much greater extent than TRPC6 (which was more apparent in the Golgi apparatus than in the plasma membrane). siRNA suppress-
ion of caveolin-1 did not significantly alter the staining pattern for the receptors or their relative expression as detected by immunofluorescence (Fig. 2).

Western blot studies (Fig. 3) and densitometric analysis (Fig. 4) of caveolar-enriched ASM plasma membrane fractions confirmed the finding of caveolin-1 by immunocytochemical studies. Caveolin-2 was also expressed, but to a lesser extent than caveolin-1, whereas caveolin-3 was undetectable by Western blot. Furthermore, all three agonist receptors, as well as STIM1, were present within the caveolar fractions, whereas TRPC6 was not (Fig. 3). siRNA suppression resulted in a ∼70% decrease in caveolin-1 expression in whole ASM cell homogenates (Figs. 3 and 4). Expression of ACh, histamine, or bradykinin receptors (as determined by Western analysis) was inconsistently decreased (e.g., decreased bradykinin, but not ACh, receptor expression).

\[ \text{[Ca}^{2+}]_{i} \] responses to agonist. In ASM cells, baseline \([\text{Ca}^{2+}]_{i}\) was 90–125 nM (99 ± 11 nM). In control (i.e., non-siRNA-transfected) ASM cells, exposure to 1 μM ACh resulted in a characteristic “biphasic” \([\text{Ca}^{2+}]_{i}\) response, with an initial, significantly higher peak followed by a sustained elevation significantly above baseline (Fig. 5). Exposure to 10 mM CD for 1 h resulted in a slow and significant increase in baseline \([\text{Ca}^{2+}]_{i}\), but only in 30% of cells. Subsequent exposure to 1 μM ACh resulted in significantly smaller peak and plateau than control \([\text{Ca}^{2+}]_{i}\) responses (Figs. 5 and 6).

In comparison to the CD effect, siRNA suppression of caveolin-1 resulted in a considerably larger decrease in peak and plateau \([\text{Ca}^{2+}]_{i}\) responses (Figs. 5 and 6). In cells exposed to Lipofectamine (vehicle control) alone, \([\text{Ca}^{2+}]_{i}\) responses were not significantly different from untreated control cell responses. For CD and caveolin-1 siRNA, interference with caveolin-1 resulted in a greater decrease in plateau than in peak \([\text{Ca}^{2+}]_{i}\) responses.

Fig. 2. Relative expression of caveolin isoforms and \([\text{Ca}^{2+}]_{i}\)-regulatory proteins in human ASM. A: semiquantitative fluorescence measurements of immunostained human ASM cell samples showing significantly weaker expression of caveolin-2 than caveolin-1 and undetectable expression of caveolin-3. Suppression of caveolin-1 expression using small interference RNA (siRNA) resulted in a significant decrease in plasma membrane staining of caveolin-1 in ASM cells. B: with caveolin-1 suppression, expression of ACh, histamine, or bradykinin receptors was inconsistently affected, but stromal interaction molecule-1 (STIM1) was decreased. *Significantly different from caveolin-1 (P < 0.05). #Significant siRNA effect.
In nontransfected ASM cells, 10 μM histamine and 10 nM bradykinin produced transient \([\mathrm{Ca}^{2+}]_i\) elevations. As with ACh, exposure to CD for 1 h decreased \([\mathrm{Ca}^{2+}]_i\) responses to both agonists. Bradykinin produced the most pronounced effect of caveolin-1 siRNA on peak or plateau responses, histamine produced an intermediate response, and ACh produced the weakest response \((P < 0.05; \text{ Figs. 5 and 6}).\)

**DISCUSSION**

Caveolae and caveolins have been found in a variety of smooth muscle types, including ASM. Studies have suggested that caveolae contain \([\mathrm{Ca}^{2+}]_i\)-regulatory proteins and, thus, may play a role in \([\mathrm{Ca}^{2+}]_i\) regulation in smooth muscle. In the present study, we have demonstrated the presence of caveolin-1 in the plasma membrane of human ASM and its colocalization with receptors of several bronchoconstrictor agonists. Furthermore, we have demonstrated that disruption of caveolae by CD, as well as inhibition of caveolin-1 expression via siRNA, significantly disrupts the \([\mathrm{Ca}^{2+}]_i\) responses to these agonists. Caveolae (and caveolin-1) also appear to be important in regulation of SOCE in ASM, not necessarily by incorporation of putative SOCE channels but, rather, by alteration of STIM1 triggering of SOCE. These data highlight the importance of caveolae and caveolin-1 in \([\mathrm{Ca}^{2+}]_i\) regulation in human ASM.

**Caveolae and caveolin expression in smooth muscle.** Caveolae, 50- to 100-nm flask-shaped invaginations at the plasma membrane identified more than 50 years ago \((37, 57)\), are enriched in cholesterol, sphingolipids, and integral membrane proteins (caveolins). Three mammalian isoforms of caveolin protein \((21–24\ \text{kDa})\) have been identified: caveolin-1, caveolin-2, and caveolin-3 \((8, 14, 21, 44, 47, 53, 54)\). Caveolin-2 is usually associated with caveolin-1. Caveolins form homooligomers and caveolin-enriched domains capable of binding other proteins via caveolin scaffolding domains. Such structural formations likely underlie the ability of caveolins to regulate signaling mechanisms at the plasma membrane. Furthermore, caveolar invaginations can allow for facilitated interactions between plasma membrane-regulatory components and intracellular structures. In this regard, it is tempting to imagine caveolae as rudimentary t tubules of striated muscle.

There is considerable heterogeneity in the expression of caveolin isoforms not only across cell types, but also across different species. In general, caveolin-1 and caveolin-2 are widely expressed, whereas caveolin-3 appears to be expressed mostly in muscle \((8, 46, 50, 54)\). Indeed, only smooth muscle appears to express all three isoforms. Such is the case in

![Fig. 3](image-url)  
**Fig. 3.** Expression of caveolin isoforms and \([\mathrm{Ca}^{2+}]_i\)-regulatory proteins in human ASM cells. A: Western blot analyses confirming immunofluorescence data (Figs. 1 and 2) demonstrating expression of caveolin-1 in whole cell fractions of ASM cells. B: much greater expression of caveolin-1 in the caveolar fraction than in intracellular (Golgi) fractions. Caveolin-2 was expression was weaker than caveolin-1 expression, and caveolin-3 was undetectable. Receptors for ACh, histamine, and bradykinin and STIM1 (trigger for SOCE) were expressed to a much greater extent in the caveolar fraction than in the Golgi fraction. siRNA suppression significantly decreased caveolin-1 expression but only slightly altered receptor expression (A).

In nontransfected ASM cells, 10 μM histamine and 10 nM bradykinin produced transient \([\mathrm{Ca}^{2+}]_i\) elevations. As with ACh, exposure to CD for 1 h decreased \([\mathrm{Ca}^{2+}]_i\); responses to both agonists. Bradykinin produced the most pronounced effect of caveolin-1 siRNA on peak or plateau responses, histamine produced an intermediate response, and ACh produced the weakest response \((P < 0.05; \text{ Figs. 5 and 6}).\)

**SOCE.** As described previously \((3)\), SOCE was triggered in ASM by first depleting SR \([\mathrm{Ca}^{2+}]_i\) stores via 1 μM CPA in the absence of extracellular \([\mathrm{Ca}^{2+}]_i\). Reintroduction of extracellular \([\mathrm{Ca}^{2+}]_i\) after SR \([\mathrm{Ca}^{2+}]_i\) depletion resulted in SOCE (Fig. 7). In control (nontransfected) cells, the amplitude of SOCE was 45% of the CPA response. Exposure to CD significantly reduced the absolute, as well as the relative (to CPA response), magnitude of the SOCE. Similarly, siRNA transfection also resulted in considerable reduction of SOCE (Fig. 7).

**DISCUSSION**

Caveolae and caveolins have been found in a variety of smooth muscle types, including ASM. Studies have suggested that caveolae contain \([\mathrm{Ca}^{2+}]_i\)-regulatory proteins and, thus, may play a role in \([\mathrm{Ca}^{2+}]_i\) regulation in smooth muscle. In the present study, we have demonstrated the presence of caveolin-1 in the plasma membrane of human ASM and its colocalization with receptors of several bronchoconstrictor agonists. Furthermore, we have demonstrated that disruption of caveolae by CD, as well as inhibition of caveolin-1 expression via siRNA, significantly disrupts the \([\mathrm{Ca}^{2+}]_i\) responses to these agonists. Caveolae (and caveolin-1) also appear to be important in regulation of SOCE in ASM, not necessarily by incorporation of putative SOCE channels but, rather, by alteration of STIM1 triggering of SOCE. These data highlight the importance of caveolae and caveolin-1 in \([\mathrm{Ca}^{2+}]_i\) regulation in human ASM.

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![Fig. 4](image-url)  
**Fig. 4.** Densitometric analysis of coexpression of caveolar proteins in ASM. As exemplified in Western blots of Fig. 3, caveolin-1 coexpressed with several \([\mathrm{Ca}^{2+}]_i\)-regulatory proteins in ASM cells. siRNA suppression of caveolin-1 slightly decreased caveolin-2 levels (but caveolin-3 remained undetectable; A), Caveolin-1 and caveolin-2, as well as receptors for ACh, histamine (H1R), and bradykinin (BKR), and putative channels (TRPC) for SOCE and STIM1 were expressed to a much greater extent in the caveolar fraction than in the Golgi fraction of ASM cells.
vascular smooth muscle cells, with all isoforms assuming a predominantly plasma membrane distribution (19, 51). However, even here, caveolin-3 expression is significantly weaker and more variable than caveolin-1 or caveolin-2 expression (11). For example, in the hamster, caveolin-1 is expressed in arterial and venous smooth muscle, whereas caveolin-3 expression is limited to the arterial smooth muscle (49). In this regard, there is no information on the expression of different caveolin isoforms in ASM. Nonetheless, a few studies have certainly established that cultured (immortalized) human ASM cells (15, 18), as well as caveolae-enriched membranes from canine ASM (10), express caveolin-1.

In the present study, we demonstrated that human ASM expresses caveolin-1. We further found that caveolin-2 is also expressed by these ASM cells (although to a lesser extent than caveolin-1). However, using fluorescence immunochemistry or Western blots, we did not detect caveolin-3 in human ASM cells. To rule out any confounding effect of cell isolation on caveolin-3 expression, we performed other pilot studies using freshly dissected human bronchial smooth muscle samples; however, caveolin-3 levels remained undetectable. We did not specifically examine caveolin-3 mRNA expression, and it is therefore possible that human ASM may express this isoform under certain conditions. However, because of the overwhelming expression of caveolin-1, it is unlikely that caveolin-3 plays a significant role at least in [Ca$_{2+}$]$_i$ regulation of ASM cells under normal conditions.

**Caveolins and [Ca$_{2+}$]$_i$ regulation.** Using caveolin-knockout mice, several laboratories examined the role of caveolin isoforms in regulation of vascular (1, 12, 14, 19, 26, 28, 44, 48) and urogenital (55) smooth muscle contraction. Caveolin-1-knockout mice develop severe pulmonary hypertension (12, 44). Because of the relative paucity of information on caveolin isoforms in ASM, much less is known about their regulation of contractility. Other studies have suggested a role for caveolin-1 in coordination of mechanisms involved in smooth muscle contractility (6, 16). For example, in vascular smooth muscle, feedback of myogenic tone is regulated by linking spontaneous transient outward currents to local Ca$_{2+}$ release in perimembranous SR (i.e., Ca$_{2+}$ sparks) (25, 32). Whereas Ca$_{2+}$ sparks are normally confined to a small (1-2-μm-diameter) intracellular area, they can activate Ca$_{2+}$ release from neighboring ryanodine receptors (Ca$_{2+}$-induced Ca$_{2+}$ release). In this regard, caveolar invaginations increase the probability of approx-

![Fig. 5. Effect of methyl-β-cyclodextrin (CD) and caveolin-1 suppression on [Ca$_{2+}$]$_i$ responses of ASM cells to agonist stimulation. In control cells, ACh (1 μM), histamine (10 μM), and bradykinin (10 nM) typically produced an initially higher (peak) [Ca$_{2+}$]$_i$, response followed by a sustained response at a lower level (plateau). Disruption of caveolae by exposure of ASM cells to CD for 1 h resulted in a significant decrease in peak and plateau [Ca$_{2+}$]$_i$, responses to all 3 agonists (each to a different extent). siRNA suppression of caveolin-1 produced even greater decreases in these responses than CD.](image-url)
imation between plasma membrane channels and SR proteins (29), thus affecting the spatial and temporal features of Ca\(^{2+}\) sparks. Indeed, spontaneous outward currents in vascular smooth muscle in caveolin-1-knockout mice are reduced, suggesting that caveolae are necessary for efficient [Ca\(^{2+}\)]\(_i\) regulation (12). These data in vascular and other smooth muscle types lay the foundation for the idea that [Ca\(^{2+}\)]\(_i\)-regulatory proteins interact by virtue of their presence in caveolae.

Given the elegant concepts developed in vascular smooth muscle, it would be certainly reasonable to suggest that caveolae facilitate agonist responses in ASM. However, much less is known about caveolae, caveolins, and [Ca\(^{2+}\)]\(_i\) regulation in ASM than in vascular smooth muscle. Recent elegant studies by Daniel and colleagues (9) in canine ASM established that caveolae-enriched membrane fractions express caveolin-1, binding proteins for L-type Ca\(^{2+}\) channels, and plasma membrane Ca\(^{2+}\)-ATPase and coprecipitate sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase and inositol trisphosphate receptor type 1 protein. Although interpretation of coprecipitation results is not straightforward, these structural studies nonetheless lay the foundation for regulation of agonist responses by caveolae and caveolin-1 in ASM. Daniel and colleagues suggest that caveolae can act as Ca\(^{2+}\) buffers for the perimembranous SR, allowing for ASM contraction to persist, even under Ca\(^{2+}\)-free conditions. Although this has been demonstrated in interstitial cells of Cajal of the intestine (9), studies in ASM are lacking.

In ASM, SR Ca\(^{2+}\) release occurs via inositol trisphosphate receptor (7), as well as ryanodine receptor (27), channels. We previously demonstrated that, similar to vascular smooth muscle, ASM also displays Ca\(^{2+}\) sparks (35, 36). However, in contrast to vascular smooth muscle, Ca\(^{2+}\) sparks may not necessarily modulate ASM membrane potential but, nonetheless, are involved in spatiotemporal integration of agonist responses (35, 36). Furthermore, the initial [Ca\(^{2+}\)]\(_i\) response to agonists such as Ach, and even histamine, largely involves SR Ca\(^{2+}\) release; however, maintenance of [Ca\(^{2+}\)]\(_i\) (i.e., a plateau phase above baseline) involves sustained Ca\(^{2+}\) influx, as well as continued SR Ca\(^{2+}\) (27, 40). The present study is the first to demonstrate that caveolae (specifically caveolin-1) are involved in regulation of [Ca\(^{2+}\)]\(_i\) in ASM, consistent with the proposal by Daniel and colleagues (9). The decreased [Ca\(^{2+}\)]\(_i\) response to agonist after acute depletion of plasma membrane cholesterol via CD suggests that caveolae are indeed present in human ASM cells and that caveolae are involved in [Ca\(^{2+}\)]\(_i\) regulation under normal conditions. Suppression of caveolin-1 via siRNA demonstrates the importance of this isoform in [Ca\(^{2+}\)]\(_i\) regulation. Overall, the results

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**Fig. 6.** Summary of effects of CD and caveolin-1 siRNA on [Ca\(^{2+}\)]\(_i\) responses. CD-induced caveolar disruption significantly decreased peak and plateau [Ca\(^{2+}\)]\(_i\) responses to histamine (10 \(\mu\)M) and bradykinin (10 nM), but to a lesser extent for ACh (1 \(\mu\)M). Effects of caveolin-1 siRNA were qualitatively similar to CD but were significantly greater than CD. Values are means ± SE. *Significantly different from control (\(P < 0.05\)). %Significantly different from CD.

![Graph](https://via.placeholder.com/150)

**Fig. 7.** Effect of CD and caveolin-1 siRNA on SOCE in ASM cells. A: in the absence of extracellular Ca\(^{2+}\), depletion of intracellular Ca\(^{2+}\) stores by exposure to cyclopiazonic acid (CPA) resulted in an elevation of [Ca\(^{2+}\)]\(_i\) levels. Subsequent reintroduction of extracellular Ca\(^{2+}\) resulted in a further increase in [Ca\(^{2+}\)]\(_i\), representing Ca\(^{2+}\) influx via SOCE. Disruption of caveolae by CD, as well as siRNA suppression of caveolin-1, significantly reduced the extent of SOCE-mediated Ca\(^{2+}\) entry, but not the response to CPA (A and B). Values are means ± SE. *Significantly different from control (\(P < 0.05\)). %Significantly different from CD.
Ca\textsuperscript{2+} influx, which may serve to maintain SR Ca\textsuperscript{2+} stores, is known to occur in ASM via voltage-gated (56) and receptor-gated (24, 31) channels, as well as in response to SR Ca\textsuperscript{2+} depletion (i.e., SOCE) (3, 23, 34). SOCE, in turn, may be mediated via TRPC. In previous studies, we established that ASM cells express multiple TRPC isoforms, including TRPC3, TRPC4, and TRPC6 (3). Accordingly, caveolae may be expected to contain several [Ca\textsuperscript{2+}]-regulatory proteins, thus facilitating plasma membrane-SR interactions. For example, in vascular smooth muscle, TRPC1, TRPC3, and TRPC4 (putative SOCE proteins) associate with caveolae via interactions with caveolin-1 (2, 4, 5). Furthermore, TRPC1 is functionally coupled to endothelin receptors in caveolae of arterial smooth muscle. Disruption of caveolae increases sensitivity of TRPC1 to SOCE and endothelin responses (5), suggesting increased Ca\textsuperscript{2+} influx in the absence of caveolin-1. However, these results appear to be the opposite of studies examining L-type Ca\textsuperscript{2+} channels and caveolae, where no effect of caveolin-1 was observed (29). In the present study, we did not find significant caveolar expression of TRPC6, which is known to be expressed in ASM (3). On the other hand, we found that TRPC4, which is also known to be expressed in ASM, does colocalize to caveolae. Thus it is possible that only certain SOCE mechanisms may be expressed in caveolae. Functionally, this would imply that, even in the absence (or decreased expression) of caveolae (e.g., with CD or caveolin-1 siRNA exposure), SOCE may not be completely inhibited (indeed, as shown in our studies). The relative importance of TRPC isoform expression in caveolae vs. noncaveolar domains remains to be established.

Recent studies have demonstrated that STIM1 acts as a Ca\textsuperscript{2+}-sensing protein that can potentially mediate communication between the SR and plasma membrane in triggering SOCE in response to depletion of SR Ca\textsuperscript{2+} stores (13, 20, 30, 33, 39, 42, 58). Although some studies (58) suggest that insertion of STIM1 from the SR to the plasma membrane is a prerequisite for SOCE activation, others have shown that STIM1 may not necessarily be inserted into the plasma membrane, but rather, might aggregate close enough to activate SOCE channels (42). Regardless of the translocation, STIM1 would be an ideal candidate caveolar protein for facilitation of plasma membrane-SR interactions. There is recent evidence for expression of STIM1 and its regulation of SOCE in ASM (39). In the present study, we found significant STIM1 expression in the caveolar fraction of human ASM cells. Suppression of caveolin-1 decreased plasma membrane expression of STIM1. Accordingly, we hypothesize that caveolar regulation of SOCE may be mediated via alterations in STIM1 expression and function after SR Ca\textsuperscript{2+} depletion.

In addition to effects mediated by altered Ca\textsuperscript{2+}-regulatory protein expression, caveolae and caveolin-1 may regulate intracellular signaling pathways. For example, Halayko and Stelmack (18) recently reported that, in human ASM cells, disruption of caveolae by CD or caveolin-1 knockdown via siRNA causes p42/p44 MAPK activation and increased cell proliferation. Caveolin-1 expression also appears to be greater in ASM cells of a contractile phenotype than in proliferating cells. Caveolin-1 siRNA inhibited increased mitogen-associated protein kinase activation, which usually occurs with exposure to platelet-derived growth factor. Recently, Hunter and Nixon (22) reported that lipid rafts and caveolin-1 are essential for TNF-\alpha-induced activation of certain signaling pathways (RhoA/Rho kinase). Although previous studies focused on cell proliferation, [Ca\textsuperscript{2+}i] regulation can also be affected by these pathways; this aspect of caveolar regulation in ASM remains to be examined.

In conclusion, the results of the present study establish the importance of caveolae and the caveolin-1 isoform in regulation of [Ca\textsuperscript{2+}i], responses to agonist in human ASM. Figure 8 is a schematic representation of the proposed role of caveolae and caveolin-1 in ASM [Ca\textsuperscript{2+}i] regulation. Future studies will focus on the role of caveolins in altered [Ca\textsuperscript{2+}i] regulation during airway inflammation and during development.

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