Caveolae facilitate muscarinic receptor-mediated intracellular Ca\(^{2+}\) mobilization and contraction in airway smooth muscle

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Gosens R, Stelmack GL, Dueck G, Mutawe MM, Hinton M, McNeill KD, Paulson A, Dakshinamurti S, Gertshoffer WT, Thliveris JA, Unruh H, Zaagsma J, Halayko AJ. Caveolae facilitate muscarinic receptor-mediated intracellular Ca\(^{2+}\) mobilization and contraction in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 293: L1406–L1418, 2007. First published September 21, 2007; doi:10.1152/ajplung.00312.2007.—Contractile responses of airway smooth muscle (ASM) determine airway resistance in health and disease. Caveolae microdomains in the plasma membrane are marked by caveolin proteins and are abundant in contractile smooth muscle in association with nanospots involved in Ca\(^{2+}\) homeostasis. Caveolae-1 can modulate localization and activity of signaling proteins, including trimeric G proteins, via a scaffolding domain. We investigated the role of caveolae in contraction and intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) mobilization induced by ACh. Human and canine ASM tissues and cells predominantly express caveolin-1. Muscarinic M\(_3\) receptors (M\(_3\)R) and G\(_\alpha_{q/11}\) colocalize with caveolin-1-rich membranes of ASM tissue. Caveolae disruption with β-cyclodextrin in canine tracheal strips reduced sensitivity but not maximum isometric force induced by ACh. In fura-2-loaded canine and human ASM cells, exposure to methyl-β-cyclodextrin (mβCD) reduced sensitivity but not maximum [Ca\(^{2+}\)]\(_i\) induced by ACh. In contrast, both parameters were reduced for the partial muscarinic agonist, pilocarpine. Fluorescence microscopy revealed that mβCD disrupted the colocalization of caveolae-1 and M\(_3\)R, but [N-methyl-\(^{3}H\)]scopolamine receptor-binding assay revealed no effect on muscarinic receptor availability or affinity. To dissect the role of caveolin-1 in ACh-induced [Ca\(^{2+}\)]\(_i\) flux, we disrupted its binding to signaling proteins using either a cell-permeable caveolin-1 scaffolding domain peptide mimetic or by small interfering RNA knockdown. Similar to the effects of mβCD, direct targeting of caveolin-1 reduced sensitivity to ACh, but maximum [Ca\(^{2+}\)]\(_i\) mobilization was unaffected. These results indicate caveolae and caveolin-1 facilitate [Ca\(^{2+}\)]\(_i\) mobilization leading to ASM contraction induced by submaximal concentrations of ACh.

Caveolin; G protein-coupled receptor; asthma; histamine; G\(_\alpha_{q/11}\)

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(48) observed that ileum longitudinal muscle had reduced contraction to endothelin-1 with no change in response to 5-HT or carbachol, whereas femoral arterial muscle contraction was increased in response to α1-adrenergic receptor stimulation. Collectively, these observations point to agonist- and tissue-specific role for caveolae and caveolin-1 in modulating receptor-mediated smooth muscle contraction.

In contractile smooth muscle cells, caveolae distribution is highly ordered, being concentrated in longitudinal plasma membrane arrays that lie in close proximity to intracellular sarcoplasmic reticulum and mitochondria, thereby forming nanospaces for Ca\(^{2+}\) homeostasis (17, 18, 40). Ultrastructural studies confirm this spatial orientation in airway smooth muscle (ASM; Ref. 31), and biochemical fractionation of caveolae from canine tracheal is revealed that caveolae are enriched in number of Ca\(^{2+}\)-handling proteins, including 1-type Ca\(^{2+}\) channels, the Ca\(^{2+}\)-binding proteins calsequestrin and calreticulin, and the plasma membrane Ca\(^{2+}\) pump (9). In a recent electrophysiological study using isolated rat cerebral resistance arterioles, Kamishima et al. (29) showed that caveolin-1 inhibition with CSD peptide or selective antibodies markedly slowed Ca\(^{2+}\) removal rate after stimulation to physiologically relevant cytoplasmic Ca\(^{2+}\) concentrations. Collectively, these studies suggest that effects of caveolae disruption or caveolin-1 depletion on GPCR-mediated smooth muscle contraction may be underpinned, in part, by changes in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) flux.

Despite the association of Ca\(^{2+}\)-handling proteins with caveolae in ASM and compelling evidence from other smooth muscle tissues that GPCR-mediated contraction is modulated by caveolae and caveolin-1, the role of caveolae in functional excitation-contraction coupling in ASM has not yet been reported. Therefore, in the current study, we tested the hypothesis that caveolin and caveolin-1 modulate [Ca\(^{2+}\)]\(_i\), mobilization and associated contractile responses induced by physiologically relevant agonists in human and canine ASM. We used biochemical fractionation of cells and tissues to assess subcellular distribution of muscarinic M3 receptors (M3R) and G\(_{q/11}\) subunits that transduce ligand-induced cell responses. We also used cholesterol depletion, small interfering RNA (siRNA) knockdown of caveolin-1, and treatment with cell-permeable caveolin-1 CSD peptide to determine the functional role of caveolae and caveolin-1 in muscarinic receptor-mediated Ca\(^{2+}\) flux in primary cultured ASM and contraction of intact tracheal smooth muscle (TSM). Our results are significant, as they describe for the first time a novel role for caveolae and caveolin-1 in ASM contractile responses, and thus they are of relevance to understanding mechanisms that control airway resistance in health and disease.

MATERIALS AND METHODS

Cell culture. Canine and human ASM cells were used for cell culture studies. Primary cultured canine airway myocytes were established from dissociated canine tracheal as we (25, 37) have described previously. Human bronchial smooth muscle cell lines immortalized by stable expression of human telomerase reverse transcriptase (htERT) were prepared as described previously (21). The primary cultured human bronchial smooth muscle cells used to generate hTERT ASM cells were prepared from macroscopically healthy segments of 2nd- to 4th generation main bronchi obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma.

All procedures were approved by the Human Research Ethics Board of the University of Manitoba.

Cells were grown to confluence using DMEM supplemented with 10% fetal bovine serum, 50 U/ml streptomycin, and 50 μg/ml penicillin. Cultures were maintained in a humidified incubator at 37°C-5% CO\(_2\), and media was changed every 2 days. For the induction of contractile phenotype myocytes, confluent cultures of canine (passages 0 or 1) or human ASM cell (passages 9-15) were serum-deprived for 4–10 days using Ham’s F-12 medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium (ITS) as described previously (21, 25).

Isolation of caveolae-enriched membrane fractions. Cells grown on uncoated 150-mm dishes were washed with ice-cold PBS and lysed in 500 mM sodium carbonate (pH 11.0) supplemented with 1 mM Na\(_2\)VO\(_4\), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, and 1 mM PMSE. After homogenization by sonification, 2 ml of homogenate was mixed with an equal volume of a solution containing 150 mM NaCl, 25 mM MES (pH 6.5), and 80% (wt/vol) sucrose and placed in the bottom of a centrifuge tube. A stepwise sucrose density gradient (65%, 50%, 30%, 20%, and 5%) was then carefully layered on top of the homogenate. Thereafter, the samples were centrifuged at 210,000 g for 18 h, and then 1-ml fractions were collected from the top of the gradient. Samples were stored at −80°C until further use.

Preparation of total protein lysates from ASM tissue. Intact ASM tissue was isolated from human bronchial specimens or canine tracheal by microdissection at 4°C. Thereafter, tissues were homogenized by sonication in ice-cold in RIPA buffer and subjected to centrifugation (760 g, 5 min), and the supernatant was stored at −80°C for subsequent protein assay and immunoblot analyses.

Western blot analysis. Protein content in supernatant samples was determined using the Bio-Rad protein assay with BSA as a reference (Bio-Rad, Hercules, CA). Equal amounts of protein from sucrose density-isolated fractions or total protein lysates were subjected to electrophoresis, transferred to nitrocellulose membranes, and analyzed for the proteins of interest using specific primary and horseradish peroxidase (HRP)-conjugated secondary antibodies as we (25) have previously described. Bands were subsequently visualized on film using ECL reagents.

Transmission electron microscopy. The ultrastructure of intact canine tracheal was assessed. Specimens consisting of two cartilage rings with intact tracheal tissues were prepared from the cervical segments using a sharp scalpel. Specimens were incubated at 37°C in oxygenated Krebs-Henseleit solution (KH; 117.5 mM NaCl, 5.6 mM KCl, 1.18 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 1.28 mM NaH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), and 5.55 mM d-glucose, gassed with 5% CO\(_2\) and 95% O\(_2\), 37°C, pH 7.4) for 1 h in the presence or absence of β-cyclodextrin (β-CD; 10 mM), which depletes cholesterol and thereby disrupts lipid rafts including caveolae. Specimens were washed once with fresh KH buffer and fixed in PBS (pH 7.4) containing 4% paraformaldehyde-1.25% glutaraldehyde at room temperature for 48 h. Thereafter, the smooth muscle layer was removed from each ring and subjected to postfixation with 1% osmium tetroxide and embedded in LX-112 acrylic medium. Ultrathin cross-sections of the muscle tissue were then prepared, mounted onto coated grids, and stained with 1% uranyl acetate and lead citrate. Cell ultrastructure was assessed with an electron microscope at an acceleration voltage of 60–80 kV.
to disrupt caveolae. Thereafter, muscle chambers were washed out repeatedly, and muscle strips were reexposed to the same concentration of ACh used for the initial stimulation. To ensure muscle strip viability, after the final ACh exposure, chambers were washed, and muscle strips were again stimulated with isosmotic KH containing 47 mM KCl. Any muscle strips that exhibited a change of more than 15% in active tension compared with that observed with the last KCl exposure before the ACh protocol were not included for data analysis.

Immunocytochemistry. ASM cells were plated onto precoated glass coverslips in six-well culture dishes. Cells were fixed for 15 min at 4°C in cytoskeletal buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, and 5 mM glucose, pH 6.1) containing 3% paraformaldehyde. Cells were then permeabilized by incubation for 5 min at 4°C in cytoskeletal buffer containing 3% paraformaldehyde and 0.3% Triton X-100. For immunofluorescence microscopy, fixed cells were first blocked for 2 h at room temperature in Cyto-TBS buffer (20 mM Tris base, 154 mM NaCl, 20 mM EGTA, and 20 mM MgCl₂, pH 7.2) containing 1% BSA and 2% normal donkey serum. Incubation with primary antibodies occurred overnight at 4°C in Cyto-TBS containing 0.1% Tween 20 (Cyto-TBST). Incubation with FITC- or Cy5-conjugated secondary antibodies was for 2 h at room temperature in Cyto-TBST. Nuclei were stained with Hoechst 33342 nuclear stain and then incubated with 5

Cy5 by 340- and 380-nm light was converted to [Ca²⁺]i concentration. Real-time quantification of cytosolic Ca²⁺ in cultured ASM cells was performed using the Ca²⁺-sensitive ratiometric fluorescent dye fura-2 AM as we (37) have described previously. All measurements were carried out using myocytes grown on glass coverslips or chamber slides. Myocytes were washed briefly in HBSS/HEPES buffer (1.26 mM CaCl₂, 5.33 mM KCl, 0.44 mM KH₂PO₄, 0.50 mM MgCl₂·6H₂O, 0.41 mM MgSO₄·7H₂O, 138 mM NaCl, 4 mM NaHCO₃, 0.30 mM NaH₂PO₄, 5.60 mM glucose, 20 mM HEPES, pH 7.4) and then incubated for 1 h at room temperature in HBSS/HEPES buffer containing 10 mM β-CD. Controls were incubated with buffer only. The same time period in buffer only.

Measurement of [Ca²⁺]i concentration. Real-time quantification of cytosolic Ca²⁺ in cultured ASM cells was performed using the Ca²⁺-sensitive ratiometric fluorescent dye fura-2 AM as we (37) have described previously. All measurements were carried out using myocytes grown on glass coverslips or chamber slides. Myocytes were washed briefly in HBSS/HEPES buffer containing 0.1% BSA and then incubated with 5 µg/ml fura-2 AM (37°C, 1 h) in buffer supplemented with 0.01% pluronic acid. Cells were then washed three times and incubated in buffer for a further hour at room temperature to allow for fura-2 AM deesterrification. Real-time changes in [Ca²⁺]i were recorded using an Olympus LX-70 inverted epifluorescence microscope (×20 objective) coupled to a Perkin-Elmer Ultra Pix FSI CCD camera controlled by UltraView imaging software. The system was further coupled to a Sutter Instruments Lambda-10-2 filter wheel and controller with repeated 200–400 ms excitation at 340 and 380 nm; emission at 510 nm was recorded continually for up to 3 min after the addition of contractile agonists. Maximum change in [Ca²⁺]i was calculated as the average baseline value subtracted from the peak [Ca²⁺]i response to agonist. The ratio of emission at 510 nm excited by 340- and 380-nm light was converted to [Ca²⁺]i values from a calibration curve generated using calcium standards and calculated by the method of Grynkiewicz (23). For studies examining the effects of cholesterol depletion on [Ca], mobilization induced by contractile agonists, fura-2-loaded cells were incubated at room temperature for 1–1.5 h in buffer containing 10 mM β-CD or 5 mM methyl-β-cyclodextrin (mβCD). Controls cultures were incubated for the same time period in buffer only. For other experiments, before fura-2 loading, myocytes were incubated (1 h, 37°C) in HBSS/HEPES containing a cell-permeable rhodamine conjugated synthetic peptide (1 µM) that included the human CSD amino acid sequence (residues 82–101) linked to the NH₂ terminus with a 17-residue antennapedia (AT) protein transduction domain (RQIKIWFQNRKMKWKK-DGIGKAFITTFVTVKGYFRY). For control experiments, cells were incubated with a rhodamine-conjugated synthetic peptide containing AT sequence linked to the NH₂-terminal residue of a scrambled CSD sequence (RQIKIWFQNNRMRKKWKK-WGIDKAFTTSTVTKYWFRY). Muscarinic receptor-binding assay. Cells were grown on uncoated 150-mm dishes and subjected to 4 days of serum deprivation. Cultures were incubated in HEPES-HBSS for 1 h at 37°C with or without 5 mM mβCD and then washed with ice-cold PBS and lysed on ice in 25 mM Tris, 2.5 mM CaCl₂ (pH 7.4), supplemented with 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, and 1 mM PMSF. After 10 strokes in a Dounce homogenizer, the homogenate was centrifuged for 5 min (1,000 × g). The supernatant was transferred to a new tube and centrifuged for 30 min (150,000 × g). The pellet-containing membrane was resuspended by sonication and stored at −80°C until further use. Binding of [3H]scopolamine ([3H]NSM) was subsequently determined by incubating equal amounts of membrane (90 µg per sample) with [3H]NSM (0.0001–1.000 nM) for 60 min at room temperature. Nonspecific binding was determined by competing with 100 nM atropine. Bound and unbound [3H]NSM was quantified by liquid scintillation counting; maximal binding capacity (Bₘax) and dissociation constant (Kᵩ) were subsequently calculated using Scatchard analysis.

siRNA transfection. Transfection to suppress caveolin-1 protein expression was carried out as we (21) have previously described for studies in which total caveolin-1 protein abundance was reduced by 50–60% 72 h after siRNA transfection. Cells were grown to 50–70% confluence in chamber slides and transiently transfected in serum-free DMEM containing antibodies with a 21-bp, double-stranded siRNA targeted against a sequence between residues 529–589 of the caveolin-1 transcript (Qiagen, Mississauga, Ontario, Canada). Cells were transfected using 2.5 µg/ml siRNA in combination with 6 µlg/ml siRNA of RnAIfect transfection reagent (Qiagen). In all studies, control transfections were performed using a nonsilencing control 21-bp siRNA (Qiagen). Cells were washed with fresh DMEM 72 h after transfection and used immediately for experiments to assess changes in cytosolic Ca²⁺ concentration in response to ACh.

Materials. Cell culture media (DMEM and Ham’s F-12), supplements (fetal bovine serum and ITS), and antibiotics (penicillin and streptomycin) were obtained from Invitrogen. Caveolin-1 primary antibody was from BD Biosciences (San Jose, CA). Mucarcinic M₃R and Gαq/11 primary antibody was kindly provided by Dr. Jurgen Wess (National Institutes of Health, Bethesda, MD). Gq/11 primary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). FITC- and Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). β-Actin primary antibody, smooth muscle myosin heavy chain (smMHC) primary antibody, anti-rabbit, anti-mouse, and anti-goat HRP-conjugated secondary antibodies, β-CD, and mβCD were from Sigma-Aldrich (St. Louis, MO). Fura-2 AM dye and Hoechst 33342 nuclear stain were from Molecular Probes (Leiden, The Netherlands). ECL reagent was from Amersham (Oakville, Ontario, Canada). Rhodamine-conjugated synthetic CSD- and scrambled CSD-AT peptides were obtained from AnaSpec (San Jose, CA).

RESULTS

Caveolin expression and caveolaee association of mucarcinic M₃R and Gαq/11. We first used immunoblotting to characterize the distribution of caveolins expressed in isolated ASM tissue from trachea or 2nd- to 3rd generation mainstem bronchi. Figure 1A shows that caveolin-1 (~24 kDa) is the predominant isoform expressed in canine tracheal, human tracheal, and human bronchial smooth muscle. Although we were unable to detect a distinct band for caveolin-2 in canine tracheal lysates (even when the total protein loaded was increased 5-fold), caveolin-2 was abundant in human ASM lysates. In fact, three distinct bands for caveolin-2, which likely correspond to α-, β-, and γ-isofoms of decreasing molecular weight, respectively, were visible in human ASM. Caveolin-3 was undetect-
Role of caveolae in ACh-induced ASM contraction. We next tested whether the association of muscarinic M3R and Gq/11 with caveolae was of functional significance. Therefore, we measured active isometric force generated by individual canine TSM strips in response to ACh exposure both before and after incubation with the cholesterol-depleting agent β-CD. When applied at concentrations of 5–10 mM, the hydrophobic core of β-CD and its analog mβCD sequester cholesterol and thereby dramatically reduce membrane cholesterol content (30). We first used transmission electron microscopy to confirm the β-CD protocol we used led to the disruption of caveolae (Fig. 2). Although organized arrays of flask-shaped membrane invaginations characteristic of caveolae were readily apparent in control samples, we were unable to see any evidence of these structures after muscle strips had been incubated in β-CD.

We next tested whether the disruption of caveolae in canine TSM strips affected receptor-mediated contractile responses. We employed a protocol in which individual strips were first contracted with a single concentration of ACh (ACh1), and then, after repeated washes, strips were incubated for 1 h in KH buffer alone (control) or in KH buffer containing β-CD.

Available in human ASM lysates; however, a pale band (∼21 kDa) for caveolin-3 was evident for canine trachealis in blots where the total protein load was increased by fivefold compared with that used to detect caveolin-1 in the same lysates.

We next used an established method for sucrose density gradient centrifugation of carbonate buffer cell lysates to investigate the subcellular distribution of muscarinic M3R and Gq/11 in isolated canine trachealis (Fig. 1B) and human bronchial smooth muscle tissue (Fig. 1C). This technique separates caveolae-enriched membrane microdomains, as they are cholesterol-enriched and thus exhibit a light buoyant density (49). As expected, caveolae were retrieved from 5–20% and 20–30% sucrose gradient interfaces; this was confirmed using Western blotting that revealed a marked enrichment of caveolin-1 protein in fractions 4–7. Notably, the fractions in which M3R (fractions 3–7) and Gq/11 (fractions 2–8) were chiefly retrieved overlapped with caveolae-rich fractions for both canine and human ASM samples (Fig. 1). Human bronchial smooth muscle lysates also revealed that a portion of M3R and Gβγ protein was present in caveolae-deficient samples, suggesting these proteins also exist in other cellular subcompartments.
Thereafter, muscle baths were washed three times, and TSM strips were treated a second time with ACh (ACh2) of the same concentration used for the ACh1 step. This was repeated for multiple strips at ACh between $10^{-10}$–$10^{-8}$ M to construct full concentration-response curves. Figure 3A shows that β-CD treatment caused a significant rightward shift of the ACh2 response curve compared with the ACh1 curve (control) at low concentrations of agonist; this effect is reflected in the sensitivity of contractile responses to ACh (pEC50), which was markedly reduced after β-CD treatment from 6.45 ± 0.06 (0.34 ± 0.01 μM) for ACh1 to 6.09 ± 0.05 (0.80 ± 0.01 μM) for ACh2 ($P < 0.0001$). To further illustrate this effect, Fig. 3B shows contractile responses of individual TSM strips induced by $10^{-7}$ and $10^{-8}$ M ACh both before (ACh1) and after (ACh2) β-CD treatment. Active contractile force was unaffected in control strips, whereas at relatively low concentrations of ACh, each strip exhibited a variable decrease in contractile response after β-CD treatment. However, treatment with β-CD did not affect the time for the initiation of ACh-induced contractile force nor did it affect the time to reach peak force. Notably, contractile responses elicited by ACh above 1 μM were unaffected by caveolae disruption (Fig. 3A), and caveolae disruption had no effect on maximum contractile force generating capacity, as active tension elicited by 47 mM KCl-supplemented isotonic KH buffer, which was used to normalize ACh-induced contractions, was unchanged after β-CD treatment (data not shown). Collectively, these observations indicate that caveolae modulate the sensitivity of ASM tissue for ACh, as indicated by a significant change in pEC50, thus providing a significant supportive role for muscarinic receptor-mediated contractile responses at low-to-moderate concentrations of agonist.

Caveolae and ACh-induced cytosolic Ca2+ in cultured canine ASM cells. Since caveolae have been implicated in Ca2+ handling by smooth muscle cells, we tested whether disrupting caveolae affects changes in free [Ca2+]i, induced by muscarinic receptor agonists. For this purpose, we first used primary cultured canine ASM cells subjected to prolonged serum deprivation, a protocol we (37) have previously shown induces a subpopulation of fully contractile myocytes that express abundant functional, cell surface muscarinic M3R. This cell subpopulation is characterized by an elongate morphology and accumulates contractile apparatus-associated proteins, including smMHC (27). We used fluorescent immunocytochemistry to determine the effects of caveolae disruption on the distribution of caveolin-1 and M3R in contractile phenotype myocytes. In 7-to-10-day serum-deprived cultures, caveolin-1 and M3R are expressed abundantly in smMHC-enriched myocytes and arranged in longitudinal linear arrays, a pattern we (25, 26) have shown is characteristic for caveolae in contractile ASM cells in culture (Fig. 4, A and B). To confirm that linear arrays of caveolin-1 and M3R are associated with caveolar membranes, we incubated serum-deprived ASM cultures with β-CD for 1 h before fixation and cell staining. Immunocytochemistry revealed that caveolae depletion caused a dramatic
rearrangement in the typical longitudinal linear arrays of caveolin-1 and M3R in contractile myocytes (Fig. 4, C and D).

We next measured the effects of cholesterol depletion on ACh-induced changes in $[\text{Ca}^{2+}]_i$, using ratiometric fluorescence microscopy of serum-deprived myocytes loaded with fura-2. Canine TSM cells were exposed to 1 μM ACh (ACh1); thereafter, cultures were incubated (1 h) in buffer alone or in buffer containing β-CD, and then a second response to 1 μM ACh (ACh2) was recorded in the cells that had exhibited a rise in $[\text{Ca}^{2+}]_i$ during the ACh1 step (Fig. 5A). Although ACh1 induced a substantial, transient elevation in $[\text{Ca}^{2+}]_i$, ranging between 250–550 nM, caveolae disruption with β-CD suppressed responses by ~50% in the same myocytes (Fig. 5B). Despite decreasing the magnitude of peak $[\text{Ca}^{2+}]_i$, we did not detect any changes in the time to reach this peak or in the magnitude and duration of the subsequent $[\text{Ca}^{2+}]_i$ plateau after β-CD treatment. These data indicate that in canine ASM cells, caveolae play a significant role in facilitating $[\text{Ca}^{2+}]_i$ increase in response to a single concentration of ACh.

Effects of caveolae depletion on muscarinic receptor-mediated $[\text{Ca}^{2+}]_i$ flux in human ASM cells. We next used human ASM cell lines to fully characterize the effects of caveolae depletion on the concentration-response relationship between ACh and $[\text{Ca}^{2+}]_i$. For these studies, we used mβCD, as it is nearly twice as effective as β-CD in mediating cellular cholesterol release, and thus we were able to use a lesser concentration of mβCD in our more rigorous experiments measuring concentration-response characteristics for different agonists. We (21, 53, 54) have shown previously that prolonged serum deprivation of human ASM cell cultures induces contractile phenotype maturation, and our current studies confirm that these cells also acquire responsiveness to muscarinic receptor stimulation (Fig. 6, A and B). To assess the role of caveolae in muscarinic receptor-mediated responses, we compared the rise in $[\text{Ca}^{2+}]_i$ elicited by ACh between cultures that had been
preequilibrated either in buffer alone or in buffer containing mβCD (Fig. 6). In a manner that mimicked the effects of cholesterol depletion on ACh-induced contraction of canine tracheal strips, caveoleal disruption of cultured human ASM cells markedly reduced the magnitude of peak [Ca\(^{2+}\)]\(_i\) induced by low concentrations of ACh (<1 μM), but there were no effects at higher concentrations of ACh (Fig. 6A). These effects are illustrated by a significant reduction in sensitivity to ACh in mβCD-treated cultures (pEC\(_{50}\) = 6.42 ± 0.04; 0.38 ± 0.02 μM) compared with control cultures (pEC\(_{50}\) = 6.83 ± 0.06; 0.15 ± 0.01 μM; P < 0.05). Importantly, changes in active cytosolic Ca\(^{2+}\) mobilization were not due to a shift in resting baseline [Ca\(^{2+}\)]\(_i\) (control: 107.0 ± 6.0 nM; mβCD: 109.0 ± 9.0 nM; n = 100 cells in 3 cultures).

To more clearly elucidate the role of caveoleae in facilitating M3R-mediated [Ca\(^{2+}\)]\(_i\) release, we completed three additional analyses. First, as a significant muscarinic receptor signaling reserve exists in ASM stimulated with ACh (24, 36), we investigated whether this could account for the lack of effect of caveoleal depletion on [Ca\(^{2+}\)]\(_i\) responses elicited above the EC\(_{50}\) for ACh. We performed experiments using the partial muscarinic agonist, pilocarpine, which requires binding to nearly all M3R to induce a maximum response. Notably, compared with control cultures, caveoleal disruption reduced both sensitivity to pilocarpine (control: pEC\(_{50}\) = 7.96 ± 0.26; 0.091 ± 0.017 μM; mβCD: pEC\(_{50}\) = 7.89 ± 0.21; 0.079 ± 0.018 μM; P < 0.01) and the maximum active [Ca\(^{2+}\)]\(_i\) response (control: 315.64 ± 39.9 nM; mβCD: 184.3 ± 24.4 nM; P < 0.001; Fig. 6B). Second, to determine whether caveoleal disruption altered muscarinic receptor availability or avidity, we performed in vitro \(^{3}H\)NMS-binding assays using membrane fractions obtained from human ASM cultures that had been pretreated with mβCD (Table 1). Neither K\(_d\) nor B\(_{max}\) for muscarinic receptors was affected by caveoleal disruption, suggesting that reduced sensitivity to ACh-induced [Ca\(^{2+}\)]\(_i\) in mβCD-treated myocytes does not appear to stem from a loss of receptors. Last, we assessed whether the selective effect of caveoleal depletion to suppress sensitivity to ACh-induced [Ca\(^{2+}\)]\(_i\) release was unique to muscarinic receptors. We completed parallel experiments using histamine (10\(^{-7}\)–10\(^{-4}\) M) or bradykinin (10\(^{-11}\)–10\(^{-7}\) M), which induce [Ca\(^{2+}\)]\(_i\) release via G\(_\text{q/11}\)-coupled H1 and B2 receptors, respectively. Unlike the effects on ACh, for histamine (Fig. 6C), caveoleal depletion inhibited both the maximum active [Ca\(^{2+}\)]\(_i\) response (control: 649.3 ± 83.4 nM; mβCD: 484.2 ± 60.71 nM; P < 0.05) and sensitivity (control: pEC\(_{50}\) = 5.72 ± 0.27; 5.36 ± 0.23 μM; mβCD: pEC\(_{50}\) = 5.71 ± 0.25; 5.09 ± 0.22 μM; P < 0.05) in human ASM. In contrast to ACh, pilocarpine, and histamine, caveoleae did not appear to modulate bradykinin effects on [Ca\(^{2+}\)]\(_i\) (Fig. 6D), as mβCD had no significant effect on ligand sensitivity (control: pEC\(_{50}\) = 9.49 ± 0.16; 2.86 ± 0.05 μM; mβCD: pEC\(_{50}\) = 9.45 ± 0.16; 2.84 ± 0.04 μM) or maximum induced [Ca\(^{2+}\)]\(_i\) (control: 562.3 ± 27.2 nM; mβCD: 537.90 ± 30.00 nM; P < 0.05). Collectively, these data indicate that caveoleal modulate GPCR-mediated [Ca\(^{2+}\)]\(_i\) release in a ligand- and receptor-selective manner, likely independent of direct effects on receptor availability.

**Role of caveolein-1 protein in muscarinic receptor-mediated [Ca\(^{2+}\)]\(_i\) release in human ASM cells.** As caveoleae appear to facilitate [Ca\(^{2+}\)]\(_i\) release in ASM cells (Figs. 5 and 6), and caveolein-1, which can serve as a scaffold and regulatory protein for a number of important intracellular signaling proteins (8), is the principal caveoleal marker protein in ASM cells (Fig. 1), we next assessed the direct role of caveolein-1 in muscarinic receptor-mediated [Ca\(^{2+}\)]\(_i\) release in cultured human ASM cells. We used a previously established selective 

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**Fig. 5.** Loss of caveoleae reduces ACh-induced Ca\(^{2+}\) responses of canine ASM cells. A: representative tracings from a typical experiment using canine ASM cells grown to confluence on coverslips and serum-deprived in insulin-supplemented media for 7–10 days. The tracings are the mean from 4 to 6 elongate cells identified in a single field. ACh (1 μM) was applied to fura-2-loaded cells to induce an increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) (ACh1); after a subsequent washout, cells were either treated with β-CD (10 nM, 1 h) to remove membrane cholesterol or with HEPES-HBSS (vehicle) control buffer. Cells were then washed again with HEPES-HBSS and exposed for a 2nd time to 1 μM ACh (ACh2). B: the mean ratio of 2nd (ACh2) and 1st (ACh1) responses for individual cells from control and β-CD-treated cultures are shown. Data represent the means ± SE from 35 cells measured in at least 3 different experiments. ***P < 0.001 (Wilcoxon test for matched pairs).
siRNA strategy (21) to induce a 50–60% decrease in caveolin-1 protein abundance in human ASM cell cultures 3 days after siRNA transfection (Fig. 7). Figure 7B shows that, compared with cells transfected with nonsilencing control siRNA, myocytes transfected with caveolin-1 siRNA exhibited a marked suppression in peak $[Ca^{2+}]_i$, induced by concentrations of ACh (0.1 and 0.01 μM) that are near or below the EC50 determined in Fig. 6 for control conditions (0.15 μM). Pilot experiments also revealed little effect of caveolin-1 knockdown on [Ca2+]i, induced by 10 μM ACh or on baseline [Ca2+], (data not shown). These data indicate that caveolin-1 knockdown impairs muscarinic receptor-mediated cytosolic Ca2+ release in a pattern and magnitude that mimics the effects of cholesterol depletion with mβCD (Fig. 6). In additional analyses of the effects of caveolin-1 siRNA, we found loss of caveolin-1 significantly reduced the number of cells per field that exhibited an active increase in $[Ca^{2+}]_i$ in response to ACh, in particular at 0.01 μM where almost 50% fewer cells were seen to mobilize cytosolic Ca2+ above baseline values (Fig. 7C). Collectively, these results indicate a central role exists for caveolin-1 in human ASM cells to facilitate muscarinic receptor-mediated cytosolic Ca2+ release.

As the ability for caveolin-1 to regulate intracellular signaling, including Ca2+ reuptake in smooth muscle cells (29), has been linked to its unique CSD, we next examined the effects of a synthetic peptide harboring the CSD sequence, linked to the cell-permeable AT protein transduction domain, on ACh-induced [Ca2+]i release in human ASM cells (Fig. 8). Confluent, 3-day serum-deprived human ASM cultures were preincubated with rhodamine-conjugated AT-CSD or amino acid residue-linked AT-scrambled-CSD in cells (Fig. 8, A and B). AT-scrambled-CSD was without effect on baseline and ACh-induced [Ca2+]i, in all cultures. Although there was no change in baseline $[Ca^{2+}]_i$, in cells loaded with fura-2, and thereafter, changes in $[Ca^{2+}]_i$, induced by ACh (0.1–1 μM) were measured. Phase contrast and fluorescent cell imaging confirmed significant and equal loading of AT-CSD and AT-scrambled-CSD in cells (Fig. 8, A and B). AT-scrambled-CSD was without effect on baseline and ACh-induced [Ca2+]i, in all cultures. Although there was no change in baseline $[Ca^{2+}]_i$, in cells loaded with AT-CSD, the CSD sequence did cause a marked reduction in peak $[Ca^{2+}]_i$ induced by sub-EC50 concentrations of ACh (0.1 and 0.01 μM; Fig. 8, C and D). In contrast, we measured no effect of AT-CSD at concentrations of ACh (10 μM) above its EC50. Notably,
Caveolae and Excitation-Contraction Coupling in Airway Smooth Muscle

Caveolin-1 orchestrates the role of caveolae in facilitating M3R-mediated [Ca^{2+}]_i release in ASM cells. This effect is manifested at ACh concentrations equal to or less than its EC_{50} value, indicating caveolae modulate sensitivity to ACh but not maximum ASM responses. Moreover, caveolae depletion did not appear to affect muscarinic receptor availability, an intracellular, postreceptor role for caveolae in ACh-triggered signaling is suggested. Our experiments using siRNA to suppress caveolin-1 protein abundance or using cell-permeable CSD peptide strongly suggest that caveolin-1 orchestrates the role of caveolae in facilitating excitation-contraction coupling in ASM, which is manifest at ACh concentrations equal to or less than its EC_{50} value, indicating caveolae modulate sensitivity to ACh but not maximum ASM responses. The potential for caveolae to serve as foci for Ca^{2+} homeostasis in smooth muscle cells is a long-established concept stemming from early observations that caveolae contain high concentrations of Ca^{2+} (43). Large conductance Ca^{2+}-activated K^+ channels, plasma membrane Ca^{2+} pump, and TRPC class transient receptor potential channels all localize to caveolae (2, 5, 9, 16). An important role in Ca^{2+} handling is also supported by ultrastructural studies, including those in ASM, that show caveolae are in close proximity to sarcoplasmic reticulum and mitochondria (18, 31). Kamishima et al. (29) recently reported that caveolae and caveolin-1 modulate the uptake of Ca^{2+} by the sarcoplasmic reticulum in isolated arteriolar myocytes after [Ca^{2+}]_i mobilization is induced. Interestingly, cerebral resistance arterioles from caveolin-1 knockout mice exhibit a marked reduction in the frequency of spontaneous transient outward currents (STOCs; Ref. 11), possibly due to spatial separation of caveolae from the sarcoplasmic reticulum where ryanodine receptor-mediated Ca^{2+} sparks that trigger STOCs originate (3, 29, 39). Our electron microscopy analyses confirm that plasma membrane invaginations characteristic of caveolae are depleted from ASM tissue after β-CD treatment, supporting the possibility that spatial disruption of Ca^{2+} nanospaces could contribute to suppressed contractile responses to ACh. Similarly, caveolae depletion caused concomitant disorganization of caveolin-1 and MsR in ASM cells, an effect that correlated with reduced mobilization of cytosolic Ca^{2+} in response to sub-EC_{50} concentrations of ACh. It did not appear

**DISCUSSION**

This study identifies caveolin-1 as the principal caveolae marker protein expressed in ASM and demonstrates, for the first time, a direct role for caveolae and caveolin-1 in ASM responses to the physiological agonist ACh. Muscarinic M_3R and G_{α_{11}} are enriched in caveolae-containing membrane microdomains of intact ASM tissue. This association appears to underpin a highly ordered spatial distribution of M_3R that, like caveolin-1, coalesce into longitudinal linear arrays, which are concomitantly disrupted by cholesterol depletion. Notably, we show that intact caveolae microdomains facilitate ACh and histamine-induced mobilization of cytosolic Ca^{2+} in cultured contractile canine and human ASM cells and contraction of intact ASM tissue. These results provide the first functional evidence for caveolae in facilitating excitation-contraction coupling in ASM, which is manifest at ACh concentrations equal to or less than its EC_{50} value, indicating caveolae modulate sensitivity to ACh but not maximum ASM responses. Moreover, as caveolae depletion did not appear to affect muscarinic receptor availability, an intracellular, postreceptor role for caveolae in ACh-triggered signaling is suggested. Our experiments using siRNA to suppress caveolin-1 protein abundance or using cell-permeable CSD peptide strongly suggest that caveolin-1 orchestrates the role of caveolae in facilitating excitation-contraction coupling in ASM, which is manifest at ACh concentrations equal to or less than its EC_{50} value, indicating caveolae modulate sensitivity to ACh but not maximum ASM responses. The potential for caveolae to serve as foci for Ca^{2+} homeostasis in smooth muscle cells is a long-established concept stemming from early observations that caveolae contain high concentrations of Ca^{2+} (43). Large conductance Ca^{2+}-activated K^+ channels, plasma membrane Ca^{2+} pump, and TRPC class transient receptor potential channels all localize to caveolae (2, 5, 9, 16). An important role in Ca^{2+} handling is also supported by ultrastructural studies, including those in ASM, that show caveolae are in close proximity to sarcoplasmic reticulum and mitochondria (18, 31). Kamishima et al. (29) recently reported that caveolae and caveolin-1 modulate the uptake of Ca^{2+} by the sarcoplasmic reticulum in isolated arteriolar myocytes after [Ca^{2+}]_i mobilization is induced. Interestingly, cerebral resistance arterioles from caveolin-1 knockout mice exhibit a marked reduction in the frequency of spontaneous transient outward currents (STOCs; Ref. 11), possibly due to spatial separation of caveolae from the sarcoplasmic reticulum where ryanodine receptor-mediated Ca^{2+} sparks that trigger STOCs originate (3, 29, 39). Our electron microscopy analyses confirm that plasma membrane invaginations characteristic of caveolae are depleted from ASM tissue after β-CD treatment, supporting the possibility that spatial disruption of Ca^{2+} nanospaces could contribute to suppressed contractile responses to ACh. Similarly, caveolae depletion caused concomitant disorganization of caveolin-1 and MsR in ASM cells, an effect that correlated with reduced mobilization of cytosolic Ca^{2+} in response to sub-EC_{50} concentrations of ACh. It did not appear

**Fig. 7.** Small interfering RNA (siRNA) knockdown of Cav-1 reduces ACh-induced Ca^{2+} responses of human ASM cells. Cultures of human ASM cells were grown on chamber slides; at subconfluence, cultures were transfected with a Cav-1 siRNA or a nonsilencing (control) siRNA. A: Western blot analysis showing that transfection with Cav-1 siRNA markedly suppressed Cav-1 protein within 24 h, and this was maintained for at least 3 days after transfection in cultures grown in serum-free media. Nonsilencing control siRNA had no effect on Cav-1 protein abundance. Blots were also probed for β-actin as a control for equal protein loading. B: 3 days after transfection with control siRNA or Cav-1 siRNA, cells were loaded with fura-2, and after exposure to sub-EC_{50} concentrations of ACh (0.01 and 0.1 μM), changes in [Ca^{2+}]_i were measured. Cav-1 protein knockdown significantly reduced peak [Ca^{2+}], induced by ACh, whereas control siRNA had no effect. C: in cultures treated with Cav-1 or control siRNA, we also counted the number of cells that were recruited to mount an increase in [Ca^{2+}], in response to 0.01 or 0.1 μM ACh. Recruitment frequency was significantly suppressed after Cav-1 knockdown. Data for each condition shown are the means ± SE of 25–45 cells from at least 3 different human ASM cell lines. *P < 0.05 and ***P < 0.001 for Cav-1 siRNA vs. nonsilencing control siRNA transfection (unpaired Student’s t-test).
that the sarcoplasmic reticulum was compromised as cholesterol depletion was without effect on resting \([\text{Ca}^{2+}]_i\), and \([\text{Ca}^{2+}]_i\) release induced by bradykinin was refractory to caveolae deletion (Fig. 6D). Interestingly, our experiments using cell-permeable CSD peptide, which competes with binding of caveolin-1 to effector proteins but does not deplete caveolae, revealed a suppression in ACh-induced \([\text{Ca}^{2+}]_i\), that was quantitatively and qualitatively similar to that caused by cholesterol depletion. These findings indicate that, in the absence of spatial separation of caveolae from sarcoplasmic reticulum and mitochondria, caveolin-1 can play a direct role in regulating \([\text{Ca}^{2+}]_i\) release triggered by ACh. To clearly discriminate the extent to which the suppression in cytosolic \([\text{Ca}^{2+}]_i\), we observed after caveolae deletion may have been caused by spatial disruption of \([\text{Ca}^{2+}]_i\) nanospaces will require future studies using high resolution, real-time assessment of local \([\text{Ca}^{2+}]_i\) concentration, inositol trisphosphate (IP3) synthesis, and the distribution of IP3 receptor, PLCβ, and Goq/11.

In addition to being integral to the spatial organization of \([\text{Ca}^{2+}]_i\) nanospaces, caveolae and caveolin-1 may also participate in excitation-contraction coupling by sequestering and regulating effector proteins. The results of our experiments provide important new insight in this area. First, we show that both M3R and its associated G protein effector subunit, Goq/11, are sequestered to caveolae microdomains in human and canine ASM. Moreover, we show this effect is correlated with a similar suppression in contractile responses. Third, our studies with siRNA confirm that caveolin-1 mediates the facilitator role of caveolae in ACh-induced \([\text{Ca}^{2+}]_i\) release, and with cell-permeable peptides we have also established that the NH2-terminal CSD is of central importance to this effect. Collectively, these data provide functional confirmation of prior descriptive associations made between caveolae and \([\text{Ca}^{2+}]_i\) homeostasis in smooth muscle cells.

A key finding of our studies is that caveolae and caveolin-1 appear to be most important in regulating the sensitivity of ASM responses to physiologically relevant concentrations of ACh. A specific role for caveolae under physiologically relevant conditions, in which \([\text{Ca}^{2+}]_i\) rarely exceeds 400 nM, has also been revealed in studies using caveolin blocking antibodies or CSD peptides to investigate \([\text{Ca}^{2+}]_i\) reuptake by the sarcoplasmic reticulum in vascular myocytes (29). In our own experiments, ACh at concentrations below 1 μM induced \([\text{Ca}^{2+}]_i\) that was less than 400 nM, and it was only at these points that we observed a suppression in contraction and/or \([\text{Ca}^{2+}]_i\) release after cholesterol depletion or treatment with caveolin-1 siRNA or CSD peptide (Fig. 6A). Conversely, at concentrations of ACh greater than 1 μM, which induced
maximum increases in $[\text{Ca}^{2+}]$, equal to or greater than 600 nM, we observed no effect of caveolae or caveolin-1 interventions. A likely explanation for the differential effects of caveolae disruption on $\text{EC}_{50}$ and maximum response to ACh is the presence of muscarinic receptor reserve that has been documented in ASM (24, 36), rendering responses to higher ACh concentrations refractory to any reduction in coupling to $\text{G}$ proteins and/or other downstream signaling effectors. As our $[\text{H}]^{3}$-NMS-binding assays suggested there was no change in receptor number after caveolae depletion, we tested the functional effects of $\text{mGBCD}$ on pilocarpine, a partial muscarinic agonist that requires recruitment of all $\text{M}_{3}\text{R}$ to induce maximum $[\text{Ca}^{2+}]_{i}$. Unlike our observations with ACh, both the $\text{EC}_{50}$ and maximum response induced by pilocarpine were suppressed by caveolae depletion. This supports the conclusion that even though disrupting caveolae inhibits ACh $\text{EC}_{50}$, receptor reserve protects against effects on maximum ACh-induced responses. These experiments also appear to negate the possibility that divergent $\text{mGBCD}$-sensitive and -insensitive pools of $\text{M}_{3}\text{R}$ exist, as might have otherwise been predicted for receptors localized to caveolae-rich or caveolae-deficient membranes, respectively. If caveolae disruption had selective effects on only a portion of receptors, a partial agonist such as pilocarpine would display a biphasic dose-response relationship; at lower concentrations, the agonist would first use fully functional $\text{mGBCD}$-insensitive receptor populations but would then only recruit $\text{mGBCD}$-sensitive receptors when much higher concentrations were added. Figure 6B demonstrates that this is not the case. Collectively, these data suggest that caveolae and caveolin-1 are likely to have significant effects on in vivo responses of smooth muscle encircling the airways. Moreover, this provides solid rationale for future investigation of the role of caveolae and caveolin-1 in ASM function in pathological conditions such as during airway inflammation associated with bronchial asthma.

Receptor- and ligand-specific effects of caveolae and caveolins on smooth muscle contractile responses have been reported. In general, for vascular smooth muscle, caveolae and caveolin appear to facilitate contraction induced by physiological agonists (2, 12, 28, 48). There are conflicting reports of the effects of caveolae on muscarinic receptor-mediated responses in gastrointestinal smooth muscle; one study showed no change in carbachol-induced responses of tissue from caveolin-1 knock-out mice (48), whereas a recent study showed that ectopic overexpression of caveolin-1 was sufficient to restore muscarinic receptor-mediated contractions in tissue from aged rabbits (50). Our studies indicate that caveolae and caveolin-1, via its CSD domain, facilitate responses of ASM to low concentration of ACh; however, we also saw that bradykinin-induced mobilization of cytosolic $\text{Ca}^{2+}$ was resistant to caveolae disruption, whereas responses to all concentrations of histamine were suppressed by cholesterol depletion. As the receptors directly involved with mediating $\text{Ca}^{2+}$ mobilization by each of these ligands are coupled to $\text{G}_{\text{q/11}}$, our findings suggest that receptor-specific differences in caveolae regulation may exist. The refractory nature of bradykinin to caveolae depletion markedly contrasts effects on ACh- or histamine-induced $\text{Ca}^{2+}$ release. We have reported that in serum-derived canine ASM cultures, responsiveness to ACh is restricted to elongate, contractile phenotype myocytes, whereas bradykinin responses are restricted to myocytes that do not exhibit the contractile phenotype (37). As our current study shows that caveolin-1 is abundant and organized into linear arrays only in elongate, contractile myocytes, it is possible that lack of dependency of bradykinin receptors on caveolae in our cultures may relate to their more prominent functional role in noncontractile myocytes. Divergence in effects of caveolae could also be related to dynamic and variable relationships between GPCRs with caveolae, as has been demonstrated in different cells. For example, on ligand binding, $\beta_{2}$-adrenergic receptors exit caveolae in cardiomyocytes (41), endothelin $\text{ET}_{A}$ receptors remain localized to caveolae in a number of cell lines (35), and in CHO and HEK cell lines, ectopically expressed B1 and B2 bradykinin receptors translocate into caveolae (47). Perhaps even more interesting, the same report revealed that although upon binding ligand both bradykinin receptor subtypes can traffic to caveolae and activate PLC via $\text{G}_{\text{q/11}}$, B2 receptors are rapidly internalized and desensitized, whereas B1 receptors remain active and subsequently activate Ras-dependent pathways (47). Thus there is suggestive evidence that caveolae trafficking of GPCRs, which can be associated with heterologous desensitization, may play a role in defining intracellular signaling responses (14, 38, 41, 47). In light of the differences we observed for different GPCRs in response to caveolae depletion, future experiments that address this issue in ASM are clearly warranted.

A number of studies suggest a facilitator role for caveolae in GPCR-mediated contraction in vascular smooth muscle (2, 12) and in smooth muscles from the gastrointestinal tract (13), the urogenital tract (32), and the myometrium (33). Our results showing that caveolae affect the sensitivity of ASM tissue to ACh-induced contraction are consistent with these reports. Although we measured the ability of caveolae and caveolin-1 to facilitate cytosolic $\text{Ca}^{2+}$ mobilization, our studies did not directly assess other mechanisms that could also contribute to the effects of caveolins and caveolin on ASM contraction. For example, caveolin-1 reportedly facilitates the interaction of some GPCRs with $\text{G}_{	ext{q/11}}$ subunits (4), and it appears to preferentially bind GDP-$\text{G}_{	ext{q/11}}$ or $\text{G}_{	ext{i/11}}$ to its CSD (34, 35). This suggests that activated, GTP-bound $\text{G}_{	ext{q/11}}$ subunits dissociate from caveolin-1. Although we did observe localization of $\text{G}_{	ext{q/11}}$ to caveolae microdomains, we did not measure the effects of caveolae depletion or caveolin-1 knockdown on $\text{M}_{3}\text{R}$-induced $\text{G}_{	ext{q/11}}$ activation. Also of relevance to GPCR agonists, phospha tidylinositol turnover, which is induced by $\text{G}_{	ext{q/11}}$ activation of PLC$\beta_{1}$ and leads to $\text{Ca}^{2+}$ release from the sarcoplasmic reticulum, is highly compartmentalized to caveolae microdomains (6, 42). Future studies examining the effects of caveolin-1 and caveolae on ACh-induced $\text{IP}_{3}$ production in ASM may reveal more precise understanding of the mechanisms regulating $\text{Ca}^{2+}$ release and contraction. Last, the recruitment of activated RhoA and PKC$\alpha$, both of which play an important role in $\text{Ca}^{2+}$-independent contractile responses of smooth muscle, can be blocked by CSD peptides, suggesting an additional pathway to be assessed in future investigations of the role of caveolae in contractile responses of ASM to physiological agonists (51).

Our observation that muscarinic $\text{M}_{3}\text{R}$ are localized to and functionally dependent on caveolae is of significant importance. Muscarinic $\text{M}_{3}\text{R}$ play an important role in airway physiology and in the pathophysiology of asthma and COPD (22). Muscarinic $\text{M}_{3}\text{R}$ are the primary muscarinic receptor subtype
responsible for contraction of human central and peripheral ASM (45). In addition, in ASM, M3R is the only muscarinic subtype that mediates IP3 accumulation and is the primary receptor mediating ACh-induced contraction (15, 46). More recent work also indicates a prominent role for muscarinic M3R in regulating airway myocyte proliferation and maturation (19, 20). Caveolae and caveolins could modulate muscarinic receptor signaling associated with these responses and thus contribute to more chronic aspects of asthma and COPD (21).

In summary, our results reveal that muscarinic M3R and Goαq/11 are localized to caveolae in ASM. Furthermore, caveolae and caveolin-1 facilitate muscarinic receptor-mediated [Ca2+]i signaling and ASM contraction induced by physiologically relevant concentrations of agonist. These effects are mediated by the CSD of caveolin-1, are not associated with alterations in receptor availability, and thus are likely the result of orchestration of key signaling effectors such as Goαs, second messenger producing enzymes, and ion channels in caveolae. Notably, we observed that caveolae contribute differentially to the facilitation of GPCR signaling in ASM cells, suggesting that unique mechanisms related to specific receptor-ligand interactions are a determinant of the functional significance of caveolae. This indicates that caveolae and caveolin-1 may play a complex role in ASM biology and function in health and disease.

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