Muscarinic receptor-mediated bronchoconstriction is coupled to caveolae in murine airways

Heike Schlenz,1 Wolfgang Kummer,1 Gitte Jositsch,1 Jürgen Wess,2 and Gabriela Krasteva1

1Institute of Anatomy and Cell Biology, Excellence Cluster Cardio-Pulmonary System, University of Giessen Lang Center, Justus-Liebig-University Giessen, Giessen, Germany; and 2Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, U.S. Department of Health and Human Services, Bethesda, Maryland

Submitted 5 August 2009; accepted in final form 13 December 2009

Schlenz H, Kummer W, Jositsch G, Wess J, Krasteva G. Muscarinic receptor-mediated bronchoconstriction is coupled to caveolae in murine airways. Am J Physiol Lung Cell Mol Physiol 298: L626–L636, 2010. First published December 18, 2009; doi:10.1152/ajplung.00261.2009.—Cholinergic bronchoconstriction is mediated by M2 and M3 muscarinic receptors (MR). In heart and urinary bladder, MR are linked to caveolin-1 or -3, the structural proteins of caveolae. Caveolae are cholesterol-rich, omega-shaped invaginations of the plasma membrane. They provide a scaffold for multiple G protein receptors and membrane-bound enzymes, thereby orchestrating signaling into the cell interior. Hence, we hypothesized that airway MR signaling pathways are coupled to caveolae as well. To address this issue, we determined the distribution of caveolin isoforms and MR subtype M2R in murine and human airways and investigated protein-protein associations by fluorescence resonance energy transfer (FRET)-confocal laser scanning microscopy (CLSM) analysis in immunolabeled murine tissue sections. Bronchoconstrictor responses of murine bronchi were recorded in lung-slice preparations before and after caveolae disruption by methyl-β-cyclodextrin, with efficiency of this treatment being validated by electron microscopy. KC1-induced bronchoconstriction was unaffected after treatment, demonstrating functional integrity of the smooth muscle. Caveolae disruption decreased muscarine-induced bronchoconstriction in wild-type and abolished it in M2R−/− and M3R−/− mice. Thus M2R and M3R signaling pathways require intact caveolae. Furthermore, we identified a presumed skeletal and cardiac myocyte-specific caveolin isofrom, caveolin-3, in human and murine bronchial smooth muscle and found it to be associated with M2R in situ. In contrast, M2R was not associated with caveolin-1, despite an in situ association of caveolin-1 and caveolin-3 that was detected. Here, we demonstrated that M2R- and M3R-mediated bronchoconstriction is caveolin-dependent. Since caveolin-3 is directly associated with M2R, we suggest caveolin-3 as novel regulator of M2R-mediated signaling.

bronchus; caveolin; fluorescence resonance energy transfer; smooth muscle; muscarinic

REGULATION OF AIRWAY DIAMETER is under prominent control of ACh released from parasympathetic nerve fibers acting on G protein-coupled muscarinic receptors (MR) situated on airway smooth muscle cells (ASMC; Ref. 10). Five molecularly distinct MR subtypes (M1R–M5R) are known. We (38) previously identified M2R and M3R as being essential for ACh-induced constriction of murine bronchial SMC. Their stimulation leads to activation of different signaling pathways in bronchial SMC, all converging onto bronchoconstriction (3, 28). Under physiological conditions, M2R binding sites outnumber M3R binding sites to a species-dependent extent, but functionally there is always a dominance of M3R in bronchoconstriction (14). However, there is evidence that the functional relevance of M2R increases under pathological conditions. Airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma are associated with bronchial hyper-reactibility and presynaptic M2R dysfunction (4). Whether postjunctional M2R are dysfunctional as well is not known. Hence, it is of relevance to identify key regulatory molecules of the airway MR signaling pathways.

Several G protein-coupled receptors are not randomly distributed along the cell surface but aggregate at specialized membrane compartments termed “caveolae.” These are 50- to 100-nm flask-shaped invaginations of the plasma membrane, containing caveolins (Cav) as major structural proteins (33). Three Cav isoforms have been identified, Cav-1, Cav-2, and Cav-3, among which the presence of either Cav-1 or Cav-3 is essential for the formation of caveolae (33). Cav-1 is widely expressed, including ASMC (13, 19, 31), whereas Cav-3 is found primarily in striated (skeletal and cardiac) muscle and certain SMC (35). In the heart and urinary bladder, MR subtypes are functionally coupled to caveolins. In rat cardiac ventricular myocytes, Feron et al. (8) showed an agonist-dependent translocation of the M2R into caveolae where a muscarinic radioligand was coimmunoprecipitated together with Cav-3. In urinary bladder SMC, the contractile response to carbachol, a MR agonist, is decreased by 67% in Cav-1 gene-deficient mice (21).

Hence, we hypothesized that the airway MR signaling pathways are coupled to caveolae as well. To address this issue, we 1) determined the distribution of Cav-1, Cav-3, and M2R in ASMC by immunohistochemistry (IHC), Western blotting, and laser-assisted microdissection with subsequent RT-PCR; 2) assessed protein-protein associations and the molecular composition of caveolae in situ by confocal laser scanning microscopy (CLSM) and fluorescence resonance energy transfer (FRET) analysis in tissue sections subjected to double-labeling indirect immunofluorescence (17); and 3) addressed the functional role of caveolin/caveolins in bronchoconstriction by videomicroscopy of precision-cut lung slices (PCLS; Ref. 23) from wild-type, M2R- and M3R-deficient mice before and after caveolae disruption by methyl-β-cyclodextrin (MCD), a cholesterol-depleting agent (34). The efficiency of MCD treatment was validated by electron microscopy.

MATERIALS AND METHODS

Muscarine and human tissue. 1) Videomorphometry, electron microscopy, laser-assisted microdissection, and RT-PCR analysis were performed on M2R- and/or M3R-deficient (M2R−/−, M3R−/−) male mice (10–20 wk old) and their corresponding wild-type mice (M2R+/−, M3R+/−).
M3R+/−). Each line was kept under specified pathogen-free conditions. The generation of M2R−/− and M3R−/− mice has been described previously (11, 44). 2) IHC, Western blotting, and FRET-CLSM analysis were performed on male and female FVB mice (10–25 wk old, kept under normal conditions). 3) For validation of M2R antibody specificity, a 31-wk-old female M2/3R double-knockout (M2/3R−/−) mouse and a 12-wk-old male wild-type mouse with the same genetic background [129/J (25%) × 129Sve (50%) × CF1 (25%)] were used. The generation of this mutant mouse strain has been described previously (38). 4) Validation of Cav-1 and Cav-3 antibody specificity was performed on tissue from two 17- to 18-wk-old male Cav-1-deficient mice (Cav−/−/−) and two corresponding 20-wk-old wild-type mice. The generation of Cav−/−/− mice with a genetic background of C57BL/6 × 5V129 has been described previously (33). For videomorphometric analysis, mice were killed by cervical dislocation. Apart from that, all animals were killed by isoflurane (Abbott, Wiesbaden, Germany) inhalation.

The human tissue (n = 5) for PCR analysis was obtained from organ donors (n = 5) whose lung had finally not been used for transplantation because of other reasons. Samples were shock-frozen isoflurane (Abbott, Wiesbaden, Germany) inhalation. cervical dislocation. Apart from that, all animals were killed by

For videomorphometric analysis, mice were killed by cervical dislocation. Apart from that, all animals were killed by isoflurane (Abbott, Wiesbaden, Germany) inhalation.

The human tissue (n = 5) for PCR analysis was obtained from organ donors (n = 5) whose lung had finally not been used for transplantation because of other reasons. Samples were shock-frozen isoflurane (Abbott, Wiesbaden, Germany) inhalation. cervical dislocation. Apart from that, all animals were killed by isoflurane (Abbott, Wiesbaden, Germany) inhalation.

The human tissue (n = 5) for PCR analysis was obtained from organ donors (n = 5) whose lung had finally not been used for transplantation because of other reasons. Samples were shock-frozen isoflurane (Abbott, Wiesbaden, Germany) inhalation. cervical dislocation. Apart from that, all animals were killed by isoflurane (Abbott, Wiesbaden, Germany) inhalation.

The human tissue (n = 5) for PCR analysis was obtained from organ donors (n = 5) whose lung had finally not been used for transplantation because of other reasons. Samples were shock-frozen isoflurane (Abbott, Wiesbaden, Germany) inhalation. cervical dislocation. Apart from that, all animals were killed by isoflurane (Abbott, Wiesbaden, Germany) inhalation.

The human tissue (n = 5) for PCR analysis was obtained from organ donors (n = 5) whose lung had finally not been used for transplantation because of other reasons. Samples were shock-frozen isoflurane (Abbott, Wiesbaden, Germany) inhalation. cervical dislocation. Apart from that, all animals were killed by isoflurane (Abbott, Wiesbaden, Germany) inhalation.
is the fluorescence intensity of the donor before photobleaching of the acceptor. The increase of fluorescence (ΔIF) for Cav-1/Cav-3, Cav-1/M2R, and Cav-3/M2R staining was measured in bronchi and heart atrium of 4–6 mice in at least 10 different ROI each. Heart atrium was applied as a positive control for the detection of association between the investigated proteins.

Laser-assisted microdissection and subsequent RT-PCR. Laser-assisted microdissection was used to isolate SMC from cryosections of tracheae and bronchioli of M3R+/− mice (n = 4) using a MicroBeam System (P.A.L.M. Microlaser Technologies, Bernried, Germany). Tissues were prepared as described for IHC analysis. Serial cryosections (6 μm) were collected on membrane slides (P.A.L.M. Microlaser Technologies) previously radiated with UV light (254 nm) for 30 min. Within 1 h after preparing the sections, equal amount of tissue was collected into the lid of cups covered with mineral oil. RNA isolation and purification were performed using RNeasy Micro Kit (Qiagen) according to the manufacturer’s protocol but omitting the DNA digestion step. Ten-microliter RNA were incubated at 70°C for 10 min; RT mix was added [2-μl 10× PCR buffer II, 2-μl MgCl2 (25 mM), 1-μl dNTPs (10 mM), 1-μl random hexamers (50 mM), 0.5-μl RNase inhibitor (20 U/μl), 1-μl murine leukemia virus RT (50 U/μl), 0.5-μl H2O; all reagents from Applied Biosystems, Darmstadt, Germany] for 30 min. Within 1 h after preparing the sections, equal amount of tissue was collected into the lid of cups covered with mineral oil. RNA isolation and purification were performed using RNeasy Micro Kit (Qiagen) according to the manufacturer’s protocol but omitting the DNA digestion step. Ten-microliter RNA were incubated at 70°C for 10 min; RT mix was added [2-μl 10× PCR buffer II, 2-μl MgCl2 (25 mM), 1-μl dNTPs (10 mM), 1-μl random hexamers (50 mM), 0.5-μl RNase inhibitor (20 U/μl), 1-μl murine leukemia virus RT (50 U/μl), 0.5-μl H2O; all reagents from Applied Biosystems, Darmstadt, Germany]. RNA was reverse-transcribed for 75 min at 43°C followed by inactivation of the RT by heating the RNA samples for 5 min at 99°C. For subsequent PCR, gene-specific intron spanning primer sets for Cav-1, Cav-3, and β-MG (Table 1) were used in mouse. Two independent primer pairs for Cav-1 and one for Cav-3 and hypoxanthine phosphoribosyltransferase (HPRT) were used in human (Table 1). The PCR conditions included initial denaturation in one cycle of 10 min at 95°C followed by 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. All analyses were done in triplicate, and the mean cycle thresholds (CT) for M2R, M3R, and β-MG were calculated. The ΔCT of M2R (ΔCTM2R) and M3R (ΔCTM3R), respectively, compared with β-MG was calculated as follows:

\[ ΔCT_{M2R} = CT_{M2R} - CT_{β-MG} \]

Control reactions included the absence of DNA template and the absence of RT. The PCR products were separated by electrophoresis on a 2% Tris-acetate-EDTA agarose gel. Specificities of the amplified human Cav-3 and HPRT PCR products were verified by sequencing (MWG Biotech, Ebersberg, Germany).

Videomorphometry. PCLS from M2R+/+, M2R−−/, M3R+/+, and M3R−−/ mice (n ≥ 4 each) were obtained by using a slightly modified version of the protocol used by Martin et al. (23) that was described in detail earlier (28, 38). In summary, the lungs were perfused with HEPES-Ringer buffer containing heparin (1,000 IU) via the right ventricle. The airways were filled via the cannulated trachea with low melting point agarose (Sigma). Lungs were dissected and cooled immediately. Two-hundred-micrometer-thick PCLS were cut (vibratome VT1000 S; Leica) from the left lobe of the lung and incubated in MEM (GIBCO, Karlsruhe, Germany) at 37°C for 2–6 h to remove the agarose. Experiments were performed in a lung-slice perfusion chamber (Hugo Sachs Elektronik, March, Germany) mounted on an inverted microscope. Images of bronchi of 150–250 μm in diameter were recorded with a charge-coupled device (CCD) camera every 60 s and analyzed with Optimas 6.5 software (Stemmer Imaging, Puchheim, Germany). The area of the airway lumen after

### Table 1. Oligonucleotide primers used in RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Acc. No.</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Product Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav-1</td>
<td>NM007616.3</td>
<td>forward GGT GAC CAA AGT TGA CG</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse AGA TGA GGT AGG GAA G</td>
<td>167</td>
</tr>
<tr>
<td>Cav-3</td>
<td>NM007617</td>
<td>forward GGT GAC CAA AGT TGA CG</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse AGA TGA GGT AGG GAA G</td>
<td>167</td>
</tr>
<tr>
<td>M2R</td>
<td>AF264049</td>
<td>forward GGT GAC CAA AGT TGA CG</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse AGA TGA GGT AGG GAA G</td>
<td>167</td>
</tr>
<tr>
<td>M3R</td>
<td>AF264050</td>
<td>forward GGT GAC CAA AGT TGA CG</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse AGA TGA GGT AGG GAA G</td>
<td>167</td>
</tr>
<tr>
<td>β-MG</td>
<td>NM009735.3</td>
<td>forward GGT GAC CAA AGT TGA CG</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse AGA TGA GGT AGG GAA G</td>
<td>167</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav-1</td>
<td>BC082246</td>
<td>forward AGG TCA ACT CGG AGG GAC AT</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CAG GTC GAT CTC TCT GGT GT</td>
<td>178</td>
</tr>
<tr>
<td>Cav-3</td>
<td>BC082246</td>
<td>forward AGG TCA ACT CGG AGG GAC AT</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CAG GTC GAT CTC TCT GGT GT</td>
<td>178</td>
</tr>
<tr>
<td>Hprt1</td>
<td>NM0001234.3</td>
<td>forward AGG TCA ACT CGG AGG GAC AT</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CAG GTC GAT CTC TCT GGT GT</td>
<td>178</td>
</tr>
</tbody>
</table>

NCBI, National Center for Biotechnology Information; Cav, caveolin; M2R and M2R, muscarinic receptor subtypes; β-MG, β-microglobulin; HPRT1, hypoxanthine phosphoribosyl-transferase 1.
10-min incubation in HEPES-Ringer buffer in the slide chamber was set as 100%. Bronchoconstriction and dilatation were expressed as a percentage decrease or increase of this area. After stimulation with muscarine, 5-HT (Sigma), and KCl, slices were washed and incubated with 10 nM MCD (Sigma) or vehicle (H2O) for 1 h at 37°C followed by restimulation with the same stimulants. Statistical analyses were performed throughout for the last minute of stimulation. Only those bronchi were included in the final data analysis, which responded to a stimulus of KCl (60 mM) with a reduction of luminal area of at least 20% in both recording series.

**Electron microscopy.** For conventional electron microscopy, PCLS from four M3R+/−/− mice were collected after videomorphometric experiments and treatment with MCD (n = 11) or vehicle (n = 9). Slices were fixed for 2–3 h in a fixative consisting of 1.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), washed in 0.1 M phosphate buffer and 3 × 5 min in 0.1 M Tris-HCl buffer, osmicated for 40 min in aqueous 1% OsO4, washed 3 × 5 min in 0.05 M maleate buffer (pH 5.2), stained en block for 1 h in 1% uranyl acetate in 0.05 M maleate buffer (pH 6.0), washed again 3 × 5 min in 0.05 M maleate buffer (pH 5.2), dehydrated in ascending concentrations of ethanol, and embedded in Epon. Sections of ~80-nm thickness were cut on an ultramicrotome (Reichert Ultracut E; Leica), stained with alkaline lead citrate and examined in an EM 902 transmission electron microscope (Zeiss).

**Fig. 1.** Protein and mRNA detection of caveolins Cav-1 and Cav-3 in murine bronchial smooth muscle cells (smc). A and C: double-labeling immunofluorescence of murine bronchi, confocal laser scanning microscopy (CLSM). A and C: Cav-1 immunoreactivity is located in the plasma membrane of bronchial smc. B and C: bronchial epithelial cells (ec) and smc display labeling for Cav-3. Smooth muscle cells also exhibit plasma membrane staining for Cav-3. A′ and B′: muscarinic receptor subtype M2R immunoreactivity is found predominantly in the plasma membrane of bronchial smc. Additionally, an unspecific labeling was detected in the cilia of the ciliated cells. A′, B′, and C′: merged images. Colocalization of the labeling results in yellow color. Arrowheads, endothelium; alv, alveolar region; lu, airway lumen. D and E: caveolin mRNA detection in laser-assisted microdissected murine airway smc (D) with subsequent RT-PCR (E) in tracheal (1) and bronchial (4 and 5) smc but not in control samples including oil (2), bronchial luminal regions proximate to the dissected smc (3), and without template (6).
RESULTS

Cav-1 and Cav-3 are present in murine and human bronchial SMC and epithelial cells. Cav-1 and Cav-3 immunoreactivities were located predominantly in the plasma membrane of murine bronchial SMC (Fig. 1, A and B). In addition, we found Cav-1 immunoreactivity in basal cells of mouse airways, as previously described (18), in vascular endothelial cells and in unidentified cells in the lamina propria, probably fibroblasts. Cav-3 immunoreactivity was detected additionally in the apical part of a subset of columnar airway epithelial cells. Furthermore, double immunostaining for M2R/Cav-1, M2R/Cav-3, and Cav-1/Cav-3 revealed colocalization of all these proteins in the plasma membrane of ASMC (Fig. 1, A′, B′, and C′).

Furthermore, the presence of Cav-1 and Cav-3 mRNA in murine ASMC was shown by laser-assisted microdissection of bronchial and tracheal SMC with subsequent RT-PCR analysis. PCR products of expected size emerged for Cav-1 and Cav-3 (Fig. 3, A-D). Omitting reactions when the RT was omitted or when no DNA template was present (Fig. 3, E) abolished the anti-Cav-3 antibody labeling. Arrows: smooth muscle cells. Bar = 50 µm. G and H: Western blotting-based validation of Cav-1 and Cav-3 antibody specificities. Bands for Cav-1α detected in wild-type mice are not present in Cav-1-deficient mice (G). H: bands corresponding to the molecular mass of Cav-3 can be preabsorbed with the corresponding antibody in a lung section from an FVB mouse (E) and a Cav-1-deficient mouse (F) is shown. Insert in E: preabsorption with the corresponding peptide abolished the anti-Cav-3 antibody labeling. Arrow: smooth muscle cells.

Statistical analysis. Nonparametric statistical tests were used. Differences between experimental and control groups in the FRET experiments and differences among mRNA expression levels in RT-PCR were analyzed with the Kruskal-Wallis test and, if P ≤ 0.05, followed by Mann-Whitney U test using SPSS software version 11.5 (SPSS Software, Munich, Germany). Data of videomorphometric experiments are presented as means ± SE of 6–11 slices obtained from 4 animals each. Matched pairs were evaluated applying Wilcoxon rank sum test. Nonmatched pairs were compared by Mann-Whitney U test. Differences were considered as statistically significant when P ≤ 0.05, P ≤ 0.01, or P ≤ 0.001.
(17), was conducted to determine whether M2R, Cav-1, and Cav-3 are in close apposition in ASMC in situ. An increase of donor fluorescence (ΔIF) after acceptor bleaching indicates an association of both proteins. A false-positive FRET signal that can be caused by cross-reactivity of secondary antibodies was excluded by applying both secondary antibodies to sections incubated with only one primary antibody. Since M2R and the caveolins are membrane proteins, we measured ΔIF in cell surface regions.

Using FRET-CLSM analysis, we confirmed the association between M2R and Cav-3 in myocytes of the heart atrium as described for cardiac ventricular myocytes by Feron et al. (8) (Supplemental Fig. S1A, available in the data supplement online at the AJPLung Cellular and Molecular Physiology web site). Furthermore, in the same set of experiments, we detected an association between M2R/Cav-3 in bronchial SMC. For M2R/Cav-3, a significant increase in M2R fluorescence in the bleached area compared with control was observed (Fig. 4). In bronchial SMC and atrial cardiac myocytes, ΔIF for Cav-1/Cav-3 was also significantly higher compared with control, implying an association between Cav-1/Cav-3 as well (Fig. 4, Supplemental Fig. S1B). No increase in donor fluores-
M3R responses to the same stimuli were tested again. PCLS from mice with MCD, a caveolae disrupting agent, and bronchoconstrictor responses to KCl in vehicle-treated PCLS from M3R mice (n = 9). We found a high number of caveolae in ASMC from vehicle-treated PCLS, mostly arranged in rows of several caveolae side by side (Fig. 5B). In PCLS treated with MCD, caveolae were completely absent from the majority of ASMC (Fig. 5C). In a few cells, their structure had only flattened, and only a very small number of structurally normal caveolae was observed.

In line with our ultrastructural observations, the bronchoconstrictor responses of the PCLS were unchanged after vehicle treatment in all mouse strains except a minimal increase in response to KCl in M2R+/+ mice (Supplemental Fig. S2). A reduction of the initial luminal area was observed at the beginning of the second recording series when PCLS were treated with MCD (P < 0.001).

After vehicle treatment, the reduction of the bronchial luminal area due to stimulation with muscarine (1 μM) was significantly stronger in M3R+/+ than in M3R−/− mice (P = 0.009; Fig. 6A). Caveolae disruption by MCD strongly decreased the contractile response to muscarine in M3R+/+ mice (P = 0.004) and completely abolished it in M3R−/− mice (P < 0.001; Fig. 6A). Interestingly, the reduction of the response to muscarine in bronchi from M3R+/+ and M3R−/− mice accounted to the same extent. The differences in maximal luminal narrowing were 37 ± 4% in M3R+/+ and 36 ± 12% in M3R−/− mice (P = 0.093).

In M2R−/− mice, the muscarine-induced bronchoconstriction was not significantly different from M2R+/+ mice (P = 0.350; Fig. 6B). In both strains, it was strongly reduced to less than 10% after MCD compared with vehicle treatment (M2R−/−: P = 0.003; M2R+/+, P < 0.001; Fig. 6B). Again, the differences in maximal luminal narrowing were nearly identical: 50 ± 5% in M2R+/+ and 54 ± 8% in M2R−/− mice (P = 0.492).

No differences in response to KCl occurred after MCD or vehicle treatment in M3R+/+ and M3R−/− mice. Bronchoconstrictor responses to KCl in vehicle-treated PCLS from M3R+/+ and M3R−/− mice were comparable (P = 1.000) and were not

![Fig. 4. Detection of close spatial association of M2R/Cav-3 and Cav-1/Cav-3 by double-labeling indirect immunofluorescence and CLSM-fluorescence resonance energy transfer (FRET) analysis in airway smooth muscle of murine bronchi in situ. Changes in donor fluorescence (ΔIF) in the membrane area of bronchial smc in experimental compared with control group are shown. For M2R/Cav-3 and Cav-1/Cav-3, ΔIF is higher in experimental groups than in controls. ***P < 0.001; *P < 0.01; n = number of regions of interest/animals. Box plots: percentiles 0, 25, median, 75, 100; extreme values (Œ).](http://ajplung.physiology.org/)

![Fig. 5. Transmission electron microscopy images of the airway wall of wild-type mice derived from precision-cut lung slices (PCLS) included in videomorphometric experiments. A: ciliated cells (cc), basal cells (ba), and secretory cells (sec) of lamina epithelialis mucosae, lamina propria with elastin (double arrowheads), and underlying tunica muscularis with bronchial smc. Bar = 1 μm. B: vehicle-treated murine ASMc containing areas with caveolae (arrows) in the plasma membrane and other subsurface vesicles (arrowheads). C: cell surface region of an equivalent bronchial smc after caveolae disruption by methyl-β-cyclodextrin (MCD). Black arrows point to plasma membrane without caveolae. nucl, Nucleus.](http://ajplung.physiology.org/)
affected by MCD treatment (M3R \textsuperscript{+/+}; P = 0.867; M3R \textsuperscript{−/−}; P = 0.505; Fig. 6A). This was also observed for M2R \textsuperscript{+/+} and M2R \textsuperscript{−/−} mice (P = 0.884; M2R \textsuperscript{+/+}; P = 0.748; M2R \textsuperscript{−/−}; P = 0.755; Fig. 6B). For 5-HT, bronchoconstrictor responses were comparable in M3R \textsuperscript{+/+} and in M3R \textsuperscript{−/−} mice after vehicle treatment (P = 0.694) and were completely abolished after MCD treatment (M3R \textsuperscript{+/+}; P = 0.006; M3R \textsuperscript{−/−}; P = 0.007; Fig. 6A). In M2R \textsuperscript{−/−} mice, responses to 5-HT were more pronounced compared with M2R \textsuperscript{+/+} mice (P = 0.007; Fig. 6B). Again, there were no statistical differences between the responses to 5-HT before and after vehicle treatment in each mouse strain (M2R \textsuperscript{+/+}; P = 0.345; M2R \textsuperscript{−/−}; P = 0.534; Supplemental Fig. S2, C and D). Responses to 5-HT were abolished in both mouse strains after MCD treatment (M2R \textsuperscript{+/+}; P = 0.002; M2R \textsuperscript{−/−}; P = 0.001; Fig. 6B).

\textbf{DISCUSSION}  

We here identified Cav-3 as a novel caveolar protein in murine and human ASMC and demonstrate its association with M2R. Accordingly, the MR-dependent component of muscarinic bronchoconstriction requires the presence of caveolae. The functional role of caveolae in MR-mediated bronchoconstriction was investigated by disruption of caveolae with MCD at a concentration comparable with that used in previously reported studies on mammalian arterial and airway SMC (5, 13, 29, 31). The efficiency of MCD treatment was confirmed by transmission electron microscopy. In line with ob-

Fig. 6. Bronchoconstrictor responses of M3R \textsuperscript{−/−} and M3R \textsuperscript{+/+} mice (A) of M2R \textsuperscript{−/−} and M2R \textsuperscript{+/+} mice (B) to stimulation with muscarine (1 \textmu M), KCl (60 mM), and 5-HT (1 \textmu M) after vehicle (---) or MCD treatment (--). Data represent luminal area with prestimulus value set as 100%. A: caveolae disruption reduces the response to muscarine in bronchi from M3R \textsuperscript{−/−} mice and fully abrogated in M3R \textsuperscript{−/−} mice. No differences in response to KCl occur after MCD or vehicle treatment in either mouse strain. 5-HT-induced responses are fully abrogated by MCD in both strains. B: in M2R \textsuperscript{−/−} and M2R \textsuperscript{+/+} mice, the muscarine-induced constriction is not significantly different from M2R \textsuperscript{−/−} mice and is reduced to less than 10% after MCD treatment. No differences in response to KCl occurred after MCD or vehicle treatment in either mouse strain. Responses to 5-HT were less distinct in M2R \textsuperscript{+/+} compared with M2R \textsuperscript{−/−} mice but were reduced after caveolae disruption in both strains. Data are presented as means ± SE; n = number of bronchi/animals.

\textbf{RT-PCR of total mRNA isolated from lung homogenates, tracheal muscle, and urinary bladder from M3R \textsuperscript{−/−} mice showed that the expression levels of M2R and M3R were comparably high in all these tissues (P = 0.222, P = 0.886, P = 0.886; Fig. 7). In M3R \textsuperscript{+/+} and M3R \textsuperscript{−/−} mice, quantification of the relative expression of M2R mRNA showed comparably high expression levels (P = 0.841 in lung, P = 0.686 in tracheal muscle, P = 0.886 in urinary bladder; Fig. 7). PCR products were of the expected size. No bands were detected in control reactions when the RT was omitted or when no DNA template was present (data not shown).}
M3R samples. Box plots: percentiles 0, 25, median, 75, 100; extreme values (outlier (n); the 5-HT-induced increase in intracellular Ca\textsuperscript{2+} coimmunoprecipitates with Cav-1 in a number of cell types (1), for subtypes 5-HT2A and 5-HT1A (45). The 5-HT2A receptor bronchoconstrictor response to 5-HT is mediated by the receptor (6, 25, 42). In mouse bronchi, however, serotonin exhibits full stimulatory effect on muscular 5-HT receptors (24, 27, 42). In human airways, the 5-HT2A subtype is expressed by prejunctional cholinergic neurons in addition to its direct functional inactivation of caveolae-coupled signaling pathways (22), and the presently observed resistance of the 5-HT-induced bronchoconstrictor response to MCD treatment further supports this notion. This intact membrane depolarization-induced contractile response excludes unspecific plasma membrane damage.

The functional role of the M3R in airway constriction is often highlighted, and bronchoconstrictor responses to methane and electrical stimulation of pulmonary vagal efferents are totally abolished in M3R\textsuperscript{−/−} mice in whole animal experiments (9). The role of M2R becomes more evident in reductionist models. A contractile response to carbachol is still present in isolated tracheal segments and smooth muscle from M3R\textsuperscript{−/−} mice, and the remaining constriction is conferred by M2R (36, 37). Accordingly, we presently observed a considerable muscarine-induced bronchoconstriction in PCLS from M3R\textsuperscript{−/−} mice, although it was significantly reduced compared with the wild-type response. These results fully confirm earlier findings, and the remaining cholinergic bronchoconstriction is mediated by the M2R (38). Notably, in our experiments, levels of M2R mRNA were not elevated in whole lung preparations and tracheal muscle in M3R\textsuperscript{−/−} mice. As several previous studies have demonstrated that the loss of one MR subtype does not affect the mRNA and protein expression level of the others (43), it is very unlikely that M2R takes over M3R function in its absence. In addition to its localization on smooth muscle, the M2R is also expressed by cholinergic neurons innervating the airway smooth muscle (10). Here, prejunctional M2R provide a negative feedback loop inhibiting ACh release (10). This inhibitory role of M2R does not become evident when PCLS are directly stimulated with muscarine, thereby bypassing neuronal ACh release. Accordingly, we observed no significant differences in the bronchoconstrictor response to muscarine (1 μM) in M2R\textsuperscript{−/−} and corresponding wild-type mice. 5-HT, in contrast, causes release of ACh from cholinergic nerve terminals in the airways in addition to its direct stimulatory effect on muscular 5-HT receptors (24, 27, 42). In the mouse trachea, the indirect, ACh-mediated effect apparently dominates over direct serotoninergic stimulation of ASMC (6, 25, 42). In mouse bronchi, however, serotonin exhibits full bronchoconstriction even in the absence of M3R (this study and Ref. 22), which argues for a predominant direct serotoninergic effect on bronchial smooth muscle. Still, we observed an augmented serotonin-induced bronchoconstriction in M2R\textsuperscript{−/−} mice, pointing toward an additional indirect, cholinergic effect with an M2R-mediated inhibitory feedback loop in this airway segment as well.

In the present study, we identified not only Cav-1, but also Cav-3 in mouse and human ASMC on mRNA and protein level. Up until now, the presence of Cav-3 in ASMC has only been described in tracheal muscle in rat (19). Whereas these findings suggest Cav-3 to be distributed throughout all mammalian species, other groups reported an absence of Cav-3 in...
isolated human ASMC (12, 13, 31). This discrepancy might be due to the different antibodies used for the detection and/or to alteration of Cav-3 expression by isolation of ASMC.

In our present experiments, caveolae disruption led to marked inhibition of the M2R- and the M3R-mediated bronchoconstriction to 1 μM muscarine in M3R<sup>−/−</sup> and M2R<sup>−/−</sup> mice, respectively, clearly demonstrating the dependence of both MR pathways on caveolae. Independently, further evidence for M2R coupling to caveolae through an association with Cav-3 is provided by double-labeling IHC combined with FRET-CLSM analysis. This is an accepted noninvasive method for investigation of protein-protein interactions in tissue sections in situ that was established and methodologically described recently (17). Nevertheless, since this method is based on indirect IHC, it does not allow to quantify extent of association and to compare this between different protein partners based on the extent of FRET effect measured. Here, we first assessed the capability of this technique to demonstrate association between Cav-3 and M2R in situ by analyzing cardiac myocytes. In rat cardiomyocytes, Cav-3 was coimmunoprecipitated with a muscarinic radioligand (8). Utilizing the FRET-CLSM approach, we demonstrated for the first time that M2R is directly associated with Cav-3 and not with Cav-1 in murine bronchial smooth muscle in situ. Collectively, the dependence of M2R-mediated bronchoconstriction on intact caveolae and the demonstrated association between Cav-3 and M2R strongly suggest that anchoring of M2R to caveolae via Cav-3 is important for initiation of M2R-mediated signaling. However, for the final proof of the functional relevance of this association, further experiments with Cav-3<sup>−/−</sup> mice are required. Unfortunately, this mouse strain was not provided to our group to perform these functional studies.

An involvement of caveolae in cholinergic airway constriction has also been proposed recently by Gosens and coworkers (10). Association, further experiments with Cav-3<sup>−/−</sup> mice are required. Unfortunately, this mouse strain was not provided to our group to perform these functional studies.

A study from Novi et al. (26) demonstrates the need of homo- or heterodimerization of MR for activation of signaling pathways via β-arrestin recruitment with M2R/M3R forming the most efficient complex. Adapting this theory, it is tempting to speculate that the formation of MR homodimers instead of heterodimers in M2R<sup>−/−</sup> and M3R<sup>−/−</sup> mice might contribute to the difference we observed in these mouse strains compared with their corresponding wild-type strains.

Both Cav-1 and Cav-3 are known to form homo- and heterooligomers with Cav-2 (2). In our Western blot analysis of murine lung homogenates using specific antibodies against Cav-1 and Cav-3, we detected 3 bands each, also suggesting formation of homooligomers in bronchial SMC. These results correspond to a previous report on Cav-1 and Cav-3 forming ~350-kDa homooligomers made of 14–16 monomers (39).

Cav-1/Cav-3 heterooligomer formation was recently shown by coimmunoprecipitation in rat and mouse cardiac myocytes and in skeletal muscle from Cav-1-overexpressing mice (2, 41). In line with these results, we detected an in situ association between Cav-1 and Cav-3 in murine ASMC using the FRET-CLSM approach. Several proteins binding to Cav-3 in muscle cells are also able to interact with Cav-1 (7, 40), suggesting that Cav-3 is exchangeable with Cav-1. Nonetheless, an association between M2R and Cav-1 was neither detected in ASMC nor in cardiac atrial myocytes in our FRET-CLSM analysis, supporting the concept that functional differences exist between the caveolin isoforms (16).

In conclusion, the present study demonstrates the presence of Cav-3 in ASMC in situ. In this tissue, it interacts with the most abundant MR subtype, M2R. Taking into consideration that M2R- and M3R-mediated bronchoconstriction is caveolae-dependent and Cav-3 is directly associated with M2R, we suggest Cav-3 as novel regulator of MR-mediated signaling.

ACKNOWLEDGMENTS

We thank Karola Michael for expert technical help with the figures.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES

1. Bhatnagar A, Sheffler DJ, Kroeze WK, Compton-Toth B, Roth BL.
   Caveolin-1 interacts with 5-HT2A serotonin receptors and profoundly
   modulates the signaling of selected Gα<sub>q</sub>-coupled protein receptors.

   M, Lee H, Frank PG, Lisanti MP. Muscle-specific interaction of caveo-
   lin isoforms: differential complex formation between caveolins in fibro-

3. Chilvers ER, Nahorski SR. Phosphoinositide metabolism in airway

4. Coulson FR, Fryer AD. Muscarinic acetylcholine receptors and airway

5. Dregja K, Voldstedlund M, Vinten J, Tranum-Jensen J, Hellstrand P,
   Sward K. Cholesterol depletion disrupts caveolae and differentially im-
   pacts agonist-induced arterial contraction. <i>Arterioscler Thromb Vasc Biol</i>

6. Eum SY, Norel X, Lefort J, Labat G, Vargafiq BB, Brink C. Ana-
   phylactic bronchoconstriction in BP2 mice: interactions between serotonin

7. Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T.
   Endothelial nitric oxide synthase targeting to caveolae. Specific interac-
   tions with caveolin isoforms in cardiac myocytes and endothelial cells.

8. Feron O, Smith TW, Michel T, Kelly RA. Dynamic targeting of the
   agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in

   mediated bradycardia and bronchoconstriction in mice lacking M2 or M3

10. Fryer AD, Jacoby DB. Muscarinic receptors and control of airway

11. Gomez J, Shannon H, Kostenis E, Felder C, Zhang L, Broddkin J,
    Grinberg A, Sheng H, Wess J. Pronounced pharmacologic deficits in M2
    muscarinic acetylcholine receptor knockout mice. <i>Proc Natl Acad Sci USA</i>