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Role of Muscarinic Receptor Subtypes in the Constriction of Peripheral Airways: Studies on Receptor-Deficient Mice

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ABSTRACT

In the airways, increases in cholinergic nerve activity and cholinergic hypersensitivity are associated with chronic obstructive pulmonary disease and asthma. However, the contribution of individual muscarinic acetylcholine receptor subtypes to the constriction of smaller intrapulmonary airways that are primarily responsible for airway resistance has not been analyzed. To address this issue, we used videomicroscopy and digital imaging of precision-cut lung slices derived from wild-type mice and mice deficient in either the M_1 (mAChR1$^{-/-}$ mice), M_2 (mAChR2$^{-/-}$ mice), or M_3 receptor subtype (mAChR3$^{-/-}$ mice) or lacking both the M_2 and M_3 receptor subtypes (mAChR2/3$^{-/-}$ double-knockout mice). In peripheral airways from wild-type mice (mAChR$^{+/+}$ mice), muscarine induced a triphasic concentration-dependent response, characterized by an initial constriction, a transient relaxation, and a sustained constriction. The bronchoconstriction was diminished by up to 60% in mAChR3$^{-/-}$ lungs and was completely abolished in mAChR2/3$^{-/-}$ lungs. The sustained bronchoconstriction was reduced in mAChR2$^{-/-}$ bronchi, and, interestingly, the transient relaxation was absent; the bronchoconstriction in response to 10^{-8} M muscarine was increased by 158% in mAChR1$^{-/-}$ mice. Quantitative reverse transcriptase-polymerase chain reaction analysis revealed that the disruption of specific mAChR genes had no significant effect on the expression levels of the remaining mAChR subtypes. These results demonstrate that cholinergic constriction of murine peripheral airways is mediated by the concerted action of the M_2 and M_3 receptor subtypes and suggest the existence of pulmonary M_1 receptor activation, which counteracts cholinergic bronchoconstriction. Given the important role of muscarinic cholinergic mechanisms in pulmonary disease, these findings should be of considerable therapeutic relevance.

Acetylcholine (ACh) released from parasympathetic nerve fibers modulates airway smooth-muscle tone via stimulation of muscarinic ACh receptors (MRs) (Zaagsma et al., 1997; Fryer and Jacoby, 1998; Barnes, 2001). An increase in pulmonary cholinergic nerve activity is associated with chronic obstructive pulmonary disease (COPD) and asthma (Zaagsma et al., 1997; Fryer and Jacoby, 1998; Barnes, 2001), and asthmatic patients are hypersensitive to the bronchoconstricting actions of muscarinic agonists (Jacoby and Fryer, 2001). Moreover, muscarinic antagonists are highly useful drugs in treating COPD and certain forms of asthma (Watson and Eglen, 1999; Jacoby and Fryer, 2001). Taken together, these findings underscore the high clinical relevance of pulmonary MRs.

Molecular cloning studies have led to the identification of five molecularly distinct MR subtypes (Caulfield and Birdsall, 1998). The M_1, M_3, and M_5 receptor subtypes couple preferentially to the $G_{q/11}$ protein, whereas the M_2 and M_4 receptors are preferentially connected with G_i (Caulfield, 1993; Felder, 1995). Stimulation of these receptors leads to a vast array of intracellular events, including the hydrolysis of phosphatidylinositol, an increase of $[Ca^{2+}]_i$, the activation of mitogen-activated protein kinases, and the inhibition of cAMP synthesis (Felder, 1995).

The M_1, M_2, and M_3 receptor subtypes have been detected in murine, porcine, and human airways (Fryer and el-Fakahany, 1990; Mak et al., 1992; Garssen et al., 1993; Hislop et al., 1998). Previous work suggests that the muscarinic constriction of tracheal smooth muscle and the main bronchi is

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ABBREVIATIONS: ACh, acetylcholine; COPD, chronic obstructive pulmonary disease; MR, muscarinic acetylcholine receptor (protein); mAChR$^{+/+}$, wild-type control (gene); mAChR2/3$^{+/+}$, wild-type control for double-knockout mice; mAChR1$^{-/-}$, muscarinic acetylcholine receptor 1-deficient mice; mAChR2$^{-/-}$, muscarinic acetylcholine receptor 2-deficient mice; mAChR3$^{-/-}$, muscarinic acetylcholine receptor 3-deficient mice; mAChR2/3$^{-/-}$, mice deficient in muscarinic acetylcholine receptors 2 and 3, PCLS, precision-cut lung slices; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; PCR, polymerase chain reaction.

mainly mediated by the M_3 receptor subtype (Roffel et al., 1990; Stengel et al., 2000, 2002). On the other hand, the contribution of individual MR subtypes to the constriction of smaller intrapulmonary airways has not been analyzed to date. However, such knowledge is of particular pathophysiological relevance because these smaller airways are primarily responsible for airway resistance (Wohlsen et al., 2001; Martin, 2002). To address this issue, we used the model of precision-cut lung slices (PCLS). This preparation allows for the study of small peripheral murine airways that are comparable in structure to the terminal bronchioles in human lung (Martin et al., 1996; Wohlsen et al., 2001). Specifically, we used videomicroscopy and digital imaging to study muscarine-mediated responses of intraparenchymal murine airways. Because multiple MRs are expressed in the lung (Zaagsma et al., 1997; Fryer and Jacoby 1998; Barnes, 2001) and it is difficult to distinguish between the different MR subtypes by classic pharmacological tools (Caulfield and Birdsall, 1998), elucidating the physiological and pathophysiological roles of the individual MR subtypes in pulmonary function represents a considerable challenge. To overcome these difficulties, we obtained PCLS preparations from recently developed MR mutant mice. To asses the functional roles of the M_1, M_2, and M_3 receptor subtypes in peripheral airway responses, we analyzed airways of different sizes in mice deficient in either the M_1 ($mAChR1^{-/-}$ mice), M_2 ($mAChR2^{-/-}$ mice), or the M_3 subtype ($mAChR3^{-/-}$ mice) (Gomeza et al., 1999; Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002). In addition, we also generated and studied mice lacking both the M_2 and M_3 subtypes ($mAChR2/3^{-/-}$ double-knockout mice).

Materials and Methods

Animals. The generation of $mAChR1^{-/-}$, $mAChR2^{-/-}$, and $mAChR3^{-/-}$ mice has been described previously (Gomeza et al., 1999; Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002). The $mAChR1^{-/-}$ and $mAChR3^{-/-}$ mice and the corresponding wild-type mice ($mAChR^{+/+}$ mice) had the following genetic background: 129SvEv (50%) × CF1 (50%). The $mAChR2^{-/-}$ mice and the corresponding wild-type mice ($mAChR2^{+/+}$ mice) had a slightly different genetic background (Gomeza et al., 1999): 129J1 (50%) × CF1 (50%).

To generate animals deficient in both the M_2 and M_3 subtypes, homozygous $mAChR2^{-/-}$ mice (Gomeza et al., 1999) were crossed with homozygous $mAChR3^{-/-}$ mice (Yamada et al., 2001; Zhang et al., 2002). The resulting F1 compound heterozygotes were then intercrossed to generate F2 mice. $mAChR2/3^{-/-}$ mice were obtained at the expected Mendelian ratio and were subsequently interbred to generate the animals used for the experiments described here. F2 wild-type mice were interbred to obtain control mice ($mAChR2/3^{+/+}$) with an equivalent genetic background [129/J1 (25%) × 129SvEv (25%) × CF1 (50%)]. All experiments were carried out with male mice that were 6 to 12 weeks old.

RT-PCR. For RT-PCR studies, lung slices from MR-deficient and wild-type mice and brains from wild-type mice ($mAChR^{+/+}$ mice; positive control) were transferred into lysis buffer (QIAGEN GmbH, Hilden, Germany) and homogenized using a mixer mill with a frequency of 300 Hz (QIAGEN). Total RNA was isolated using spin columns according to the protocol recommended by the manufacturer (RNeasy Kit, QIAGEN). Contaminating DNA was removed using DNase (1 U/μg total RNA; Invitrogen, Carlsbad, CA) in the presence of 20 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, and 50 mM KCl for 15 min at 25°C. Equal amounts of RNA were reverse-transcribed in the presence of 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, 0.5 mM dNTPs (Invitrogen), and 25 μg oligo(dT) (MWG Biotech, Ebersberg, Germany), with 200 U of Superscript RNase H^- reverse transcriptase (Invitrogen) for 50 min at 42°C. For the PCR, 2 mM $MgCl_2$ (10 mM), 0.25 mM dNTP (10 mM), 0.5 U/25 μl AmpliTaq Gold DNA Polymerase (all reagents from PerkinElmer Life Sciences, Boston, MA), and 20 μM of each primer (Table 1; MWG Biotech) were mixed (buffer 2). Cycling conditions for PCR were 10 min at 95°C, 40 cycles of 30 s at 94°C, 20 s at 62°C, and 30 s at 73°C, followed by 7 min at 73°C.

Quantitative RT-PCR. Real-time quantitative PCR (iCycler; Bio-Rad, Munich, Germany) was used to quantify levels of mAChR1, -2, and -3 mRNAs in PCLS of MR subtype-deficient lungs. Preparation of cDNA was done as described for RT-PCR. Gene-specific TaqMan PCR primers and probes specific for mouse mAChR1, -2, and -3 and GAPDH (Table 1) were designed using Primer Express software (Applied Biosystems, Foster City, CA). All PCRs were prepared in triplicate from four to eight animals using a ready-to-use kit according to the manufacturer's protocol (QuantiTect, QIAGEN). Primers specific for GAPDH were used for standardization. The data were

TABLE 1
Primers and probes for RT-PCR and qRT-PCR

Gene	Accession No.	Primer	Sequence	BasePairs
RT-PCR				
mAChR1	NM_007698	Fwd	cag tcc caa cat cac cgt ctt	441
		Rev	gag aac gaa gga aac caa cca c	
mAChR2	AF264049	Fwd	tgt ctc cca gtc tag tgc aag g	369
		Rev	cat tct gac ctg acg atc caa c	
mAChR3	AF264050	Fwd	gta caa cct cgc ctt tgt ttc c	245
		Rev	gac aag gat gtt gcc gat gat g	
GAPDH	NM_008084	Fwd	gtg atg ggt gtg aac cac gag	120
		Rev	cca cta tgc caa agt tgt ca	
		Probe	ctc aag att gtc agc aat gca tcc tgc ac	
qRT-PCR				
mAChR1	NM_007698	Fwd	ttg gca ctt tct cca tgaac	71
		Rev	ggc cag tgt gcc cag agc	
		Probe	tat acc aca tac ctg ctc atg ggc cac tg	
mAChR2	AF264049	Fwd	gct gcg tgg gtt ctt tcc t	66
		Rev	ccc cta cga tga act gcc ag	
		Probe	cct ctg ggc ccc agc cat tct ct	
mAChR3	AF264050	Fwd	cca tct ggc aag tgg tct tc	86
		Rev	tgc cac aat gac aag gat gtt g	
		Probe	ctg gcttcc tgg cat tgg tga cca tca	

qRT-PCR, real-time quantitative reverse-transcriptase polymerase chain reaction.

normalized by subtracting the threshold cycle levels between the mAChRs and GAPDH.

Videomorphometry. Mice were killed by cervical dislocation, and precision-cut lung slices were prepared using a slight modification of the protocol described by Martin et al. (1996). Briefly, the lungs were perfused via the right ventricle with Krebs-Ringer buffer containing heparin (1000 IU), penicillin/streptomycin (1%), and sodium nitroprusside (0.075 μM). The airways were filled via the cannulated trachea with agarose (low-melting-point agarose, 1.6% in Krebs-Ringer buffer; Sigma Chemie, Deisenhofen, Germany). Subsequently, the lungs and heart were removed en bloc and placed in ice-cold Krebs-Ringer buffer. The cranial lobe of the right lung was cut into 200- to 250-μm thick slices using a vibratome (VT1000S; Leica, Wetzlar, Germany). For the removal of the agarose, the slices were incubated in minimal essential medium at 37°C under normoxic conditions for 2 to 4 h. Experiments were performed under normoxic conditions in a lung-slice superfusion chamber (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) mounted on an inverted microscope (Leica) equipped with a camera (Stemmer Imaging, Puchheim, Germany). The MR agonist muscarine was purchased from Sigma Chemie. Viable airways of 100 to 400 μm in diameter were examined and incubated in the slide chamber for 5 min in minimal essential medium until the first image was acquired. The area of the airway lumen at the beginning of the experiment was defined as 100%, and bronchoconstriction or dilation was expressed as a percentage decrease or increase of this area. The studies were performed using slices after 4 to 8 h in culture. For the analysis, airways were subsequently analyzed using the Optimas 6.5 Image Analysis software program (Media Cybernetics, Silver Spring, MD).

Statistical Analysis. Data are presented as means ± S.E.M. of 4 to 18 slices obtained from three to six animals. Because it is principally impossible to test for a normal (Gaussian) distribution at such numbers, nonparametric statistical tests were used (SPSS software; SPSS Inc., Chicago, IL). Matched pairs were evaluated with use of the Wilcoxon rank sum test. In the case of more than two nonmatched groups, Mann-Whitney *U* test for comparison between two groups was conducted only when statistically significant differences were reached by the global Kruskal-Wallis test that was performed first. Differences were considered statistically significant when $p < 0.05$.

Results

RT-PCR. We initially used RT-PCR to study the expression of the mAChR1, mAChR2, and mAChR3 subtypes in murine PCLS preparations. Qualitative RT-PCR analysis revealed the expression of all three subtypes in preparations from wild-type mice (mAChR$^{+/+}$, mAChR2$^{+/+}$, and mAChR2/3$^{+/+}$ mice) (Fig. 1, B–D). As expected, no mAChR1, mAChR2, and mAChR3 signals were found with mAChR1$^{-/-}$, mAChR2$^{-/-}$, and mAChR3$^{-/-}$ mice, respectively (Fig. 1, B and C). Similarly, no mAChR2 and mAChR3 bands were observed with RNA prepared from mAChR2/3$^{-/-}$ mice (Fig. 1D), confirming the identify of the different knockout strains. We next used TaqMan analysis to quantitate and compare mAChR1, -2, and -3 mRNA levels between wild-type and mAChR mutant mice. GAPDH expression served as an internal control. These studies showed that the inactivation of specific mAChR genes had no significant effect (Kruskal-Wallis and Mann-Whitney *U* tests) on the expression levels of the remaining mAChR subtypes (Fig. 1E). Although mAChR1, -2, and -3 expression levels were generally reduced in mAChR2$^{+/+}$ mice (compared with mAChR$^{+/+}$ mice), no differences in the expression of mAChR1 and mAChR3 were observed between mAChR2$^{+/+}$ and mAChR2$^{-/-}$ mice (Fig. 1F).

Videomorphometry of Wild-Type Mice. In lung slices from wild-type mice (mAChR$^{+/+}$, mAChR2$^{+/+}$, and mAChR2/3$^{+/+}$ mice), the cumulative administration of muscarine (10^{-8}-10^{-4} M) resulted in concentration-dependent bronchoconstrictor responses (decreases in luminal airway area) (Figs. 2–4). The basal airway diameters were the following:

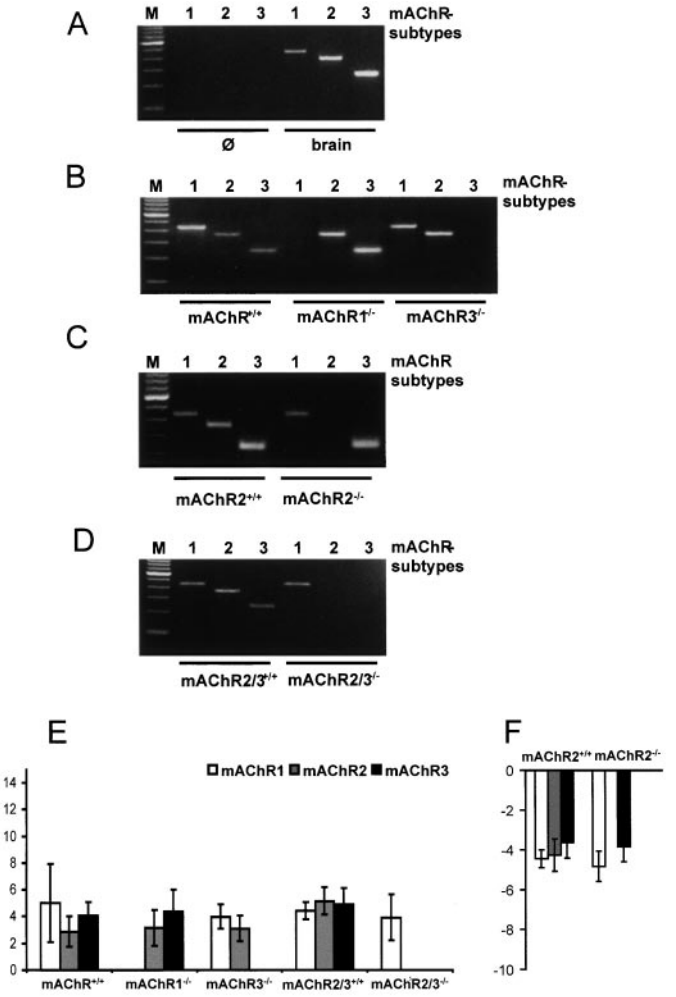

Fig. 1. RT-PCR analysis of mAChR1, -2, and -3 expression in mouse PCLS. Primers specific for the mAChR1, -2, and -3 subtypes were used to amplify cDNA prepared from mouse PCLS (B–E) and brain total RNA (A, positive control). A, mAChR1, -2, -3 expression in brain from mAChR$^{+/+}$ mice. No products were detected after omission of the template (Ø). B, mAChR1, -2, and -3 expression in PCLS from wild-type (mAChR$^{+/+}$) and mAChR1$^{-/-}$ and mAChR3$^{-/-}$ single-knockout mice. C, mAChR1–3 expression in PCLS from wild-type (mAChR2$^{+/+}$) and mAChR2$^{-/-}$ single-knockout mice. D, mAChR1, -2, and -3 expression in PCLS from wild-type (mAChR2/3$^{+/+}$) and mAChR2/3$^{-/-}$ double-knockout mice. E, quantitative RT-PCR analysis (TaqMan) revealed that disruption of individual mAChR genes had no significant effect (Kruskal-Wallis test) on the expression levels of the remaining mAChR subtypes. The data were normalized by subtracting the threshold cycle levels between the mAChRs and GAPDH. The numbers above the individual lanes in A through D indicate the mAChR subtype that was amplified. As expected, the mAChR1-, -2–, and -3–specific primers gave no signal in mice in which the respective mAChR genes (mAChR1$^{-/-}$, mAChR3$^{-/-}$, mAChR2/3$^{-/-}$) were disrupted, confirming the identity of the mutant animals. F, TaqMan analysis also showed that disruption of the mAChR2 gene had no significant effect (Kruskal-Wallis test) on the expression levels of the mAChR1 and mAChR3 subtypes. For details regarding primer sequences and RT-PCR conditions, see Table 1 and *Materials and Methods*.

mAChR$^{+/+}$, 215 ± 31 μm; mAChR2$^{+/+}$, 216 ± 13 μm; and mAChR2/3$^{+/+}$, 205 ± 24 μm. The muscarine responses consisted of three distinct phases (Figs. 2B and 5). First, a robust bronchoconstriction developed within the first minute after muscarine administration. This initial bronchoconstriction was followed by a transient relaxation response. Finally, this activity was followed by a sustained bronchoconstriction. Approximately 10 min after administration, the highest muscarine concentration used (10^{-4} M) led to a ~50 to 60% reduction of the luminal area (luminal area of 48 ± 7% in mAChR$^{+/+}$ mice, 43 ± 3% in mAChR2$^{+/+}$ mice, and 40 ± 9% in mAChR2/3$^{+/+}$ mice) (Figs. 2D, 3D, and 4D).

A single application of 10^{-4} M muscarine to smaller airways with a diameter of approximately 160 μm (mAChR$^{+/+}$, 155 ± 11 μm; mAChR2$^{+/+}$, 167 ± 7 μm) caused a pronounced bronchoconstrictor response (remaining luminal area: 16 ± 6%) (Fig. 5). The secondary transient relaxation was more pronounced in mAChR$^{+/+}$ than in mAChR2$^{+/+}$ mice (Fig. 5).

mAChR1$^{-/-}$ Mice. In mAChR1$^{-/-}$ mice (airway diameter, 277 ± 21 μm), cumulative administration of muscarine (10^{-8}-10^{-4} M) induced concentration-dependent decreases in airway area, as observed with wild-type mice (Fig. 2B). However, compared with bronchi from wild-type animals, muscarine caused significantly stronger bronchoconstriction responses in mAChR1$^{-/-}$ bronchi at concentrations of 10^{-8} M (luminal area of 69 ± 7% in mAChR1$^{-/-}$ mice versus 88 ± 6% in mAChR$^{+/+}$ mice, $p = 0.036$, Mann-Whitney U test) (Fig. 2, C and D) and 10^{-6} M (luminal area of 52 ± 8% in mAChR1$^{-/-}$ mice versus 62 ± 13% in mAChR$^{+/+}$ mice, $p = 0.021$, Mann-Whitney U test) (Fig. 2, C and D). At the highest concentration of muscarine used (10^{-4} M), however, the response of mAChR1$^{-/-}$ bronchi was not significantly different from that of mAChR$^{+/+}$ preparations (luminal area of 39 ± 9% versus 46 ± 11%, respectively; $p = 0.145$, Mann-Whitney U test) (Fig. 2, C and D). Strikingly, the transient bronchorelaxation response after the initial bronchoconstriction in wild-type preparations was absent in smaller bronchi from mAChR1$^{-/-}$ mice (airway diameter, 139 ± 12 μm) (Figs. 2B and 5).

mAChR2$^{-/-}$ Mice. In mAChR2$^{-/-}$ mice (airway diameter, 242 ± 8 μm), cumulative application of muscarine (10^{-8}-10^{-4} M) led to concentration-dependent bronchoconstriction, as was observed with the corresponding wild-type mouse strain (mAChR2$^{+/+}$ mice) (Fig. 3B). Compared with bronchi from wild-type animals, mAChR2$^{-/-}$ bronchi showed similar rapid bronchoconstriction responses (Fig. 3C) but a subsequent relaxation in the presence of 10^{-4} M muscarine (luminal area 10 min after muscarine, 69 ± 8% in mAChR2$^{-/-}$ mice versus 55 ± 5% in mAChR2$^{+/+}$ mice, $p = 0.023$, Mann-Whitney U test) (Fig. 3D). Similarly, the sustained bronchoconstriction of smaller airways, measuring 168 ± 7 μm in diameter, after a single application of 10^{-4} M muscarine was greatly reduced in mAChR2$^{-/-}$ mice (luminal area 15 min

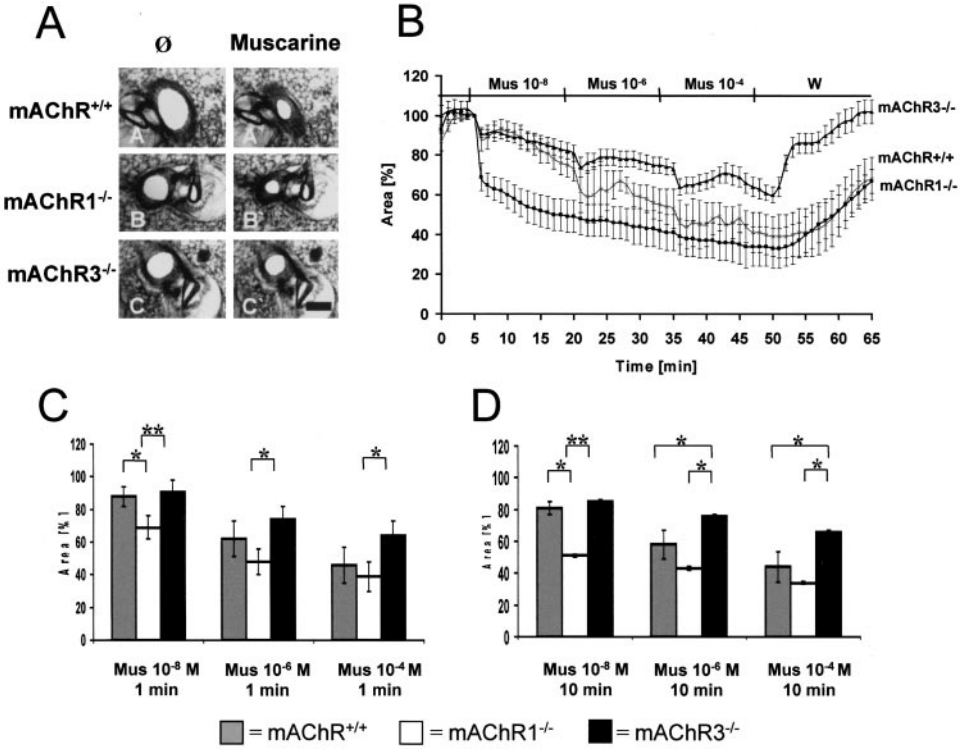

Fig. 2. Muscarine-mediated changes in the luminal area of peripheral bronchi from wild-type and mAChR1$^{-/-}$ and mAChR3$^{-/-}$ mutant mice aged 6 to 12 weeks. Changes in the luminal area of mouse peripheral airways were recorded by videomorphometry after cumulative application of different concentrations of muscarine. A, videomorphometric images of precision-cut lung slices before (Ø) and 10 min after administration of 10^{-4} M muscarine. The bronchi from wild-type (mAChR1$^{+/+}$), mAChR1$^{-/-}$, and mAChR3$^{-/-}$ mice constricted in response to 10^{-4} M muscarine. Scale bar, 250 μm. B, muscarine (Mus) induced concentration-dependent decreases in luminal airway area in wild-type (mAChR$^{+/+}$) and mAChR1$^{-/-}$ and mAChR3$^{-/-}$ single-knockout mice. Bronchi of mAChR$^{+/+}$ (nine slices from five lungs) and mAChR3$^{-/-}$ mice (13 slices from 5 lungs) responded to 10^{-8} to 10^{-4} M muscarine with an initial rapid constriction, a transient relaxation, and a sustained constriction. The transient relaxation was absent in mAChR1$^{-/-}$ lung slices (nine slices from four lungs). The bronchoconstriction response to muscarine (10^{-8} and 10^{-6} M) was increased in mAChR1$^{-/-}$ mice and was reduced in mAChR3$^{-/-}$ bronchi (10^{-6} and 10^{-4} M muscarine). W, 10-min washing step. C and D, luminal area of peripheral bronchi of mAChR$^{+/+}$ (▨), mAChR1$^{-/-}$ (□), and mAChR3$^{-/-}$ (■) mice. Measurements were taken 1 min (C) and 10 min (D) after application of 10^{-8}, 10^{-6}, and 10^{-4} M muscarine. ⋆, $p \leq 0.05$; ⋆⋆, $p \leq 0.01$, Mann-Whitney U test conducted after Kruskal-Wallis test with $p \leq 0.05$.

after muscarine, 57 ± 7% in mAChR2$^{-/-}$ mice versus 33 ± 8% in mAChR2$^{+/+}$ mice, $p = 0.049$, Mann-Whitney U test) (Fig. 5). In contrast, the initial rapid bronchoconstriction response did no differ between mAChR2$^{+/+}$ and mAChR2$^{-/-}$ mice (Fig. 5).

mAChR3$^{-/-}$ Mice. In mAChR3$^{-/-}$ mice (airway diameter, 212 ± 14 μm), cumulative muscarine application (10^{-8}-10^{-4} M) led to concentration-dependent bronchoconstriction responses, as was observed with mAChR$^{+/+}$ mice (Fig. 2B). The application of a low concentration of muscarine (10^{-8} M) led to a bronchoconstriction response that did not differ significantly from that obtained with mAChR$^{+/+}$ mice (Fig. 2, B–D). However, higher muscarine concentrations (10^{-6} M and 10^{-4} M) resulted in significantly reduced bronchoconstriction responses in mAChR3$^{-/-}$ mice (10^{-6} M muscarine: luminal area of 64 ± 5% in mAChR3$^{-/-}$ mice versus 44 ± 8% in mAChR$^{+/+}$ mice, $p = 0.029$, Mann-Whitney U test; 10^{-4} M muscarine: luminal area of 60 ± 4% in mAChR3$^{-/-}$ mice versus 46 ± 11% in mAChR$^{+/+}$ mice, $p = 0.018$, Mann-Whitney U test) (Fig. 2, B and C).

Compared with preparations from wild-type mice, single administration of 10^{-4} M muscarine to smaller airways (168 ± 11 μm in diameter) from mAChR3$^{-/-}$ mice caused a significantly reduced rapid bronchoconstriction response (by approximately 60%; luminal area of 68 ± 5 μm in mAChR3$^{-/-}$ mice versus 16 ± 6 μm in mAChR$^{+/+}$ mice, $p = 0.04$, Mann-Whitney U test) (Fig. 5). The transient relaxation response observed with preparations from mAChR3$^{-/-}$ mice (39 ± 10% relaxation after initial constriction) was not different from that seen with mAChR$^{+/+}$ mice (35 ± 10%) (Fig. 5).

mAChR2/3$^{-/-}$ Mice. We also generated (see *Materials and Methods*) and studied mAChR2/3$^{-/-}$ double-knockout mice. Strikingly, the bronchoconstriction response to the cumulative application of muscarine (10^{-8}-10^{-4} M) was completely abolished in PCLS preparations from mAChR2/3$^{-/-}$ mice (airway diameter, 192 ± 23 μm) (Fig. 4, A-D). The corresponding wild-type control mice (mAChR2/3$^{+/+}$) gave the same spectrum of responses as found with the mAChR$^{+/+}$ mice (Figs. 2 and 4).

Discussion

Muscarinic cholinergic mechanisms play a key role in the regulation of airway resistance (Zaagsma et al., 1997; Fryer and Jacoby, 1998; Barnes, 2001). Moreover, increased cholinergic activity is known to be associated with pulmonary diseases such as COPD and certain forms of asthma (Zaagsma et al., 1997; Fryer and Jacoby, 1998; Barnes, 2001). Airway resistance is determined largely by the diameter of smaller intrapulmonary airways (Martin 2002; Escolar et al., 2003). A better understanding of which MR subtypes contribute to the constriction of these intrapulmonary airways is therefore of considerable clinical relevance. To address this question, we used the PCLS model, which has been shown to maintain the integrity of all components of the peripheral lung, including viable peripheral airways (Martin et al., 1996; Wohlsen et al., 2001).

We initially demonstrated with the use of an RT-PCR strategy that the mAChR1, mAChR2, and mAChR3 subtypes are expressed in mouse peripheral airways. In agreement with this finding, these receptor subtypes are present in various other pulmonary preparations from different species, including humans (Zaagsma et al., 1997; Fryer and Jacoby, 1998; Barnes, 2001). The lack of ligands endowed with a high degree of MR subtype selectivity (except for some recently discovered snake toxins) represents a considerable obstacle

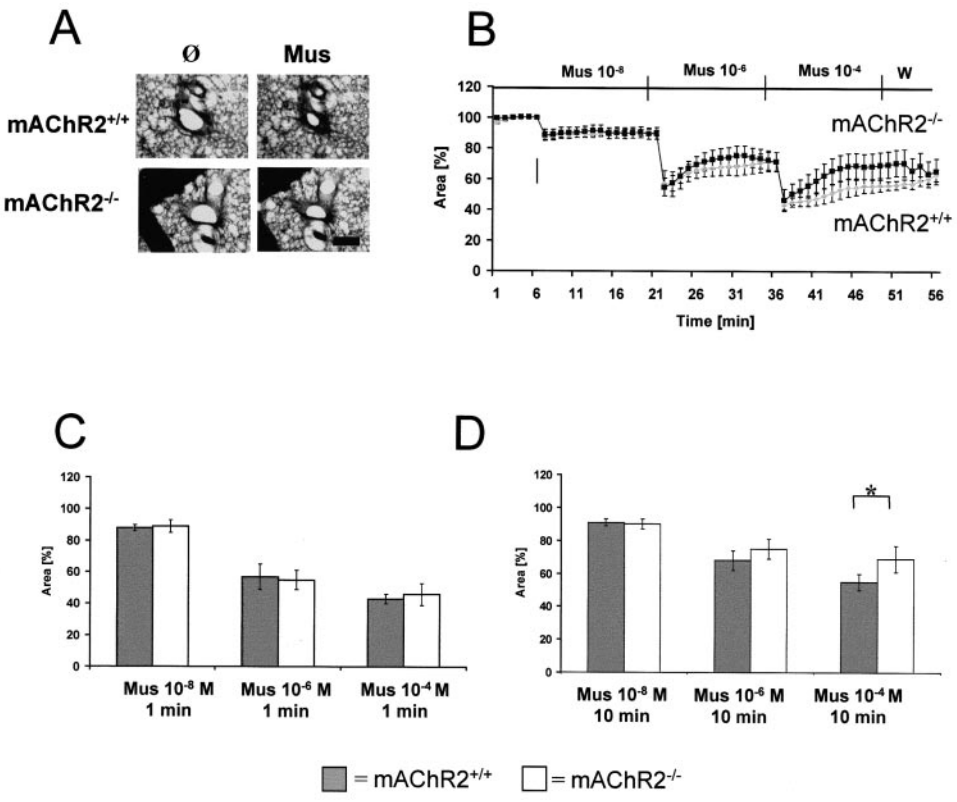

Fig. 3. Muscarine-mediated changes in the luminal area of peripheral bronchi from wild-type and mAChR2$^{-/-}$ mutant mice aged 6 to 8 weeks. A, videomorphometric images of precision-cut lung slices before (Ø) and 10 min after stimulation with 10^{-4} M muscarine (Mus). The bronchi from wild-type mAChR2$^{+/+}$ mice constricted in response to 10^{-4} M muscarine, whereas the mAChR2$^{-/-}$ bronchi dilated in the presence of the agonist. Scale bar, 250 μm. B, muscarine induced concentration-dependent decreases in luminal airway area in wild-type mAChR2$^{+/+}$ and mAChR2$^{-/-}$ single-knockout mice. Bronchi of mAChR2$^{+/+}$ mice (11 slices from 4 lungs) responded to 10^{-8} to 10^{-4} M muscarine, with an initial rapid constriction and a sustained constriction. W, 10-min washing step. In the presence of 10^{-4} M muscarine, the sustained bronchoconstriction was significantly attenuated in preparations from mAChR2$^{-/-}$ mice (10 slices from 4 lungs) ($p = 0.023$). C and D, luminal area of peripheral bronchi of mAChR$^{+/+}$ and mAChR2$^{-/-}$ mice. Measurements were taken 1 min (C) and 10 min (D) after application of 10^{-8}, 10^{-6}, and 10^{-4} muscarine. ⋆, $p \leq 0.05$; ⋆⋆, $p \leq 0.01$, Mann-Whitney U test conducted after Kruskal-Wallis test with $p \leq 0.05$.

in identifying the physiological roles of the individual MR subtypes (Caulfield and Birdsall, 1998). To examine the importance of individual MR subtypes in airway function in a more direct fashion, we therefore took advantage of the recent availability of mutant mouse strains deficient in specific mAChR subtypes. Specifically, we studied muscarine-mediated responses of mouse peripheral airways from mAChR1$^{-/-}$, mAChR2$^{-/-}$, and mAChR3$^{-/-}$ mice (Gomeza et al., 1999; Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002) and from newly generated mAChR2/3$^{-/-}$ mice. This analysis allowed us to determine the relative contributions made by the M_1, M_2, and M_3 receptor subtypes to the constriction of peripheral airways. Quantitative RT-PCR studies showed that the disruption of individual mAChR genes did not lead to compensatory changes in the expression levels of the remaining MR subtypes.

Previous pharmacological studies indicated that the M_3 receptor subtype is responsible for the receptor-mediated constriction of mouse and human trachea and human larger bronchi (Roffel et al., 1990; Stengel et al., 2000). In the present study, we observed a markedly reduced cholinergic constriction in mAChR3$^{-/-}$ bronchi of the peripheral lung similar to that observed in the trachea of the same mouse strain (Stengel et al., 2002). In the trachea, mAChR3 deletion reduced the constriction to the ACh-receptor agonist carbamylcholine by ~50% (Stengel et al., 2002), whereas the reduction amounted to up to 60% in peripheral bronchi of different sizes (this study). These findings clearly indicate that muscarinic airway constriction involves both M_3 and non-M_3 MR subtypes. Despite the general reduction in muscarine-induced bronchoconstriction in mAChR3$^{-/-}$ mice, a triphasic response, characterized by an initial constriction, a transient relaxation, and a sustained constriction, still persisted in this mutant mouse strain.

Previous studies have shown that airway smooth-muscle cells express both the M_2 and M_3 receptor subtypes (Zaagsma et al., 1997; Fryer and Jacoby 1998; Barnes, 2001). To test the hypothesis that the M_2 receptor subtype contributes to cholinergic constriction of peripheral airways, we studied muscarine-mediated airway responses in mAChR2$^{-/-}$ mice and mAChR2/3$^{-/-}$ mice. Strikingly, muscarine-mediated bronchoconstriction was completely abolished in mAChR2/3$^{-/-}$ mice. This observation clearly indicates that MR-mediated constriction of mouse peripheral airways involves the activation of both the M_2 and M_3 receptor subtypes and that no additional MR subtypes are involved. Using independently generated mAChR3$^{-/-}$ and mAChR2/3$^{-/-}$ mice, Matsui et al. (2002) recently showed that activation of both the M_2 receptor and M_3 receptor subtypes also fully accounts for muscarinic agonist-mediated contractile responses of ileal and bladder smooth-muscle preparations. This concerted action of the M_2 and M_3 receptor subtypes in regulating smooth muscle tone therefore seems to represent a rather general phenomenon.

A recent study (Matsui et al., 2003) showed that the relaxant effects of forskolin on muscarinic agonist-induced contractions were significantly greater in tracheal preparations from mAChR2$^{-/-}$ mice than in the corresponding preparations from wild-type mice, suggesting that a component of the muscarinic agonist-induced contraction response in tracheal smooth muscle involves an M_2 receptor-mediated inhibition of the relaxant effect of agents that increase cAMP levels. However, our data indicate that the M_2 receptor subtype is also directly involved in maintaining bronchoconstriction because bronchi from mAChR2$^{-/-}$ mice showed an attenuation of the sustained constriction response observed after muscarine administration. The underlying molecular pathways are still unclear but may include M_2 receptor-mediated activa-

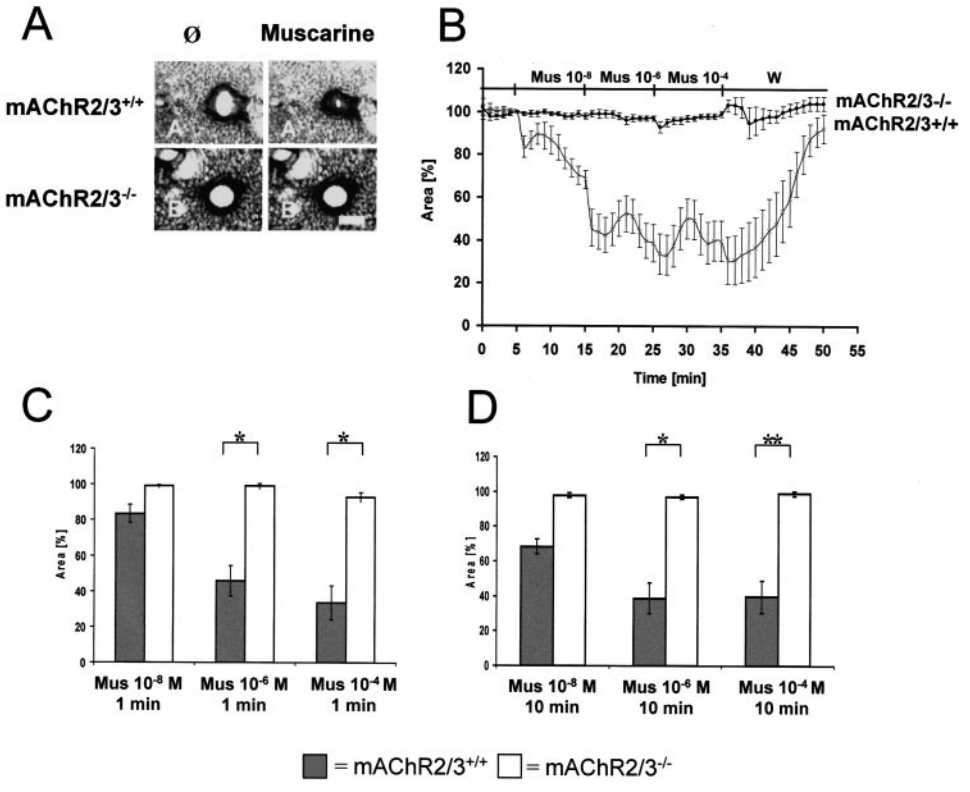

Fig. 4. Absence of muscarine-mediated constriction of peripheral bronchi from mice deficient in both M_2 and M_3 receptor subtypes aged 6 to 9 weeks. A, the bronchi of wild-type mice constricted in response to 10^{-4} M muscarine. In contrast, the luminal area of mAChR2/3$^{-/-}$ bronchi remained unchanged after muscarine treatment. Scale bar, 250 μm. B, luminal area of peripheral bronchi from wild-type mice (mAChR2/3$^{+/+}$) (nine slices from five lungs) and mAChR2/3$^{-/-}$ mice (seven slices from four lungs) after cumulative application of different concentrations of muscarine (Mus). Muscarine induced concentration-dependent decreases in luminal airway area in bronchi from wild-type mice. These responses were totally abolished in preparations from mAChR2/3$^{-/-}$ mice. W, 10-min washing step. C and D, luminal area of peripheral bronchi of wild-type mAChR2/3$^{+/+}$ (▨) and mAChR2/3$^{-/-}$ mice (□). Measurements were taken 1 min (C) and 10 min (D) after application of 10^{-8}, 10^{-6}, and 10^{-4} M muscarine. ⋆, $p \leq 0.05$; ⋆⋆, $p \leq 0.01$, Mann-Whitney U test conducted after Kruskal-Wallis test with $p \leq 0.05$.

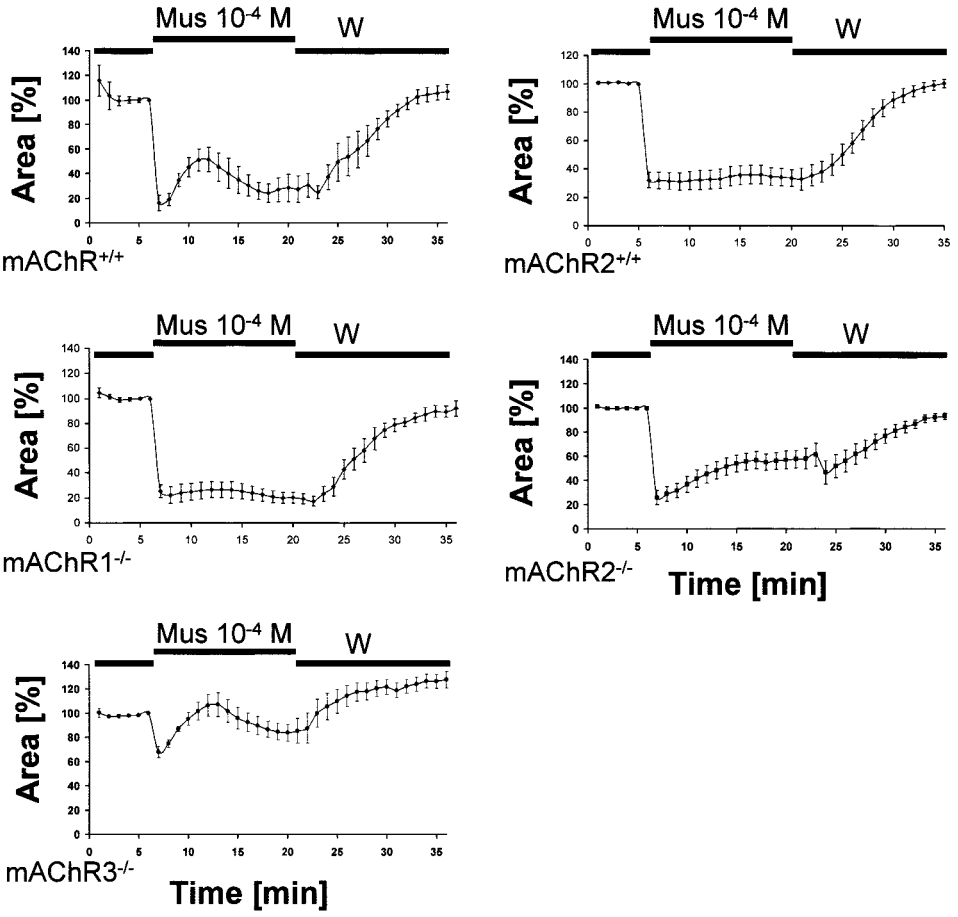

Fig. 5. Muscarine-mediated changes in luminal area of peripheral bronchi from wild-type and mAChR mutant mice aged 5 weeks. Changes in luminal area of mouse peripheral airways were recorded by videomorphometry after single application of 10^{-4} M muscarine (Mus). Muscarine induced decreases in luminal airway area in wild-type ($mAChR^{+/+}$ and $mAChR2^{+/+}$) and $mAChR1^{-/-}$, $mAChR2^{-/-}$, and $mAChR3^{-/-}$ single-knockout mice. Bronchi of $mAChR^{+/+}$ (six slices from three lungs) and $mAChR3^{-/-}$ mice (four slices from three lungs) responded to muscarine with an initial rapid constriction, a transient relaxation, and a sustained constriction. The transient relaxation was absent in $mAChR1^{-/-}$ lung slices (three slices from three lungs). The sustained constriction was reduced in small airways of $mAChR2^{-/-}$ mice (11 slices from 4 lungs) compared with bronchi from $mAChR2^{+/+}$ mice (18 slices from 6 lungs). W, 10-min washing step.

tion of Ca^{2+}-dependent potassium channels (Kotlikoff et al., 1992). The differences in airway contractility and MR expression levels observed between the $mAChR2^{+/+}$ mice and the other two wild-type strains may be related to the slightly different genetic background of the $mAChR2^{+/+}$ mice (see *Materials and Methods*).

In addition to the M_2 and M_3 receptor subtypes, the M_1 receptor is also expressed in lung tissue (Barnes 1993; Reinheimer et al., 2000). In the present study, bronchi from $mAChR1^{-/-}$ mice showed a significantly increased bronchoconstriction in response to muscarine (10^{-8} M) and lacked the transient relaxation after muscarine administration independent of the airway caliber. These data suggest the existence of an M_1 receptor-dependent pathway counteracting cholinergic bronchoconstriction, possibly via the release of a relaxing agent. Both respiratory epithelia and sympathetic nerve terminals within bronchial smooth muscle are equipped with M_1 receptors (Maclagan et al., 1989; Shapiro et al., 2001) and releasable bronchodilating agents (epithelium: nitric oxide and prostaglandin E_2) (Spicuzza et al., 2002; Tilley et al., 2003). Studies with the M_1 receptor-preferring antagonist pirenzepine have also suggested the existence of pulmonary (ganglionic) M_1 receptors modulating airway diameter (Bloom et al., 1988; Maclagan et al., 1989). The relative contributions to M_1 receptor-dependent relaxation made by epithelial cells and nerve terminals, both of which are present in the prepared PCLS, cannot be deduced from the present data.

Previous studies have shown that the M_4 receptor subtype is the predominant MR expressed in rabbit lung tissue (Lazareno et al., 1990). Moreover, work by Kilbinger et al. (1995) suggested the existence of presynaptic M_4 receptors mediating the autoinhibition of acetylcholine release in the guinea pig trachea. However, the results of this study, together with the observation that the inactivation of the mAChR4 gene in mice has no significant effect on carbachol-mediated contraction responses in tracheal smooth muscle (Stengel et al., 2000), suggest that M_4 receptors do not play a significant role in muscarinic agonist-induced bronchoconstriction, at least not in the mouse.

In conclusion, the present study demonstrates that cholinergic constriction of murine peripheral airways is mediated by the concerted action of the M_2 and M_3 receptor subtypes. Our data also strongly suggest the existence of pulmonary M_1 receptors, the activation of which counteracts this bronchoconstrictor activity. These findings should be of considerable relevance for the development of novel muscarinic drugs useful for the treatment of COPD and asthma.

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References

Barnes PJ (2001) New treatments for chronic obstructive pulmonary disease. *Curr Opin Pharmacol* **1:**217–222.
Barnes PJ (1993) Muscarinic receptor subtypes in airways. *Life Sci* **52:**521–527.
Bloom JW, Baumgartener-Folkerts C, Palmer JD, Yamamura HI, and Halonen M (1988) A muscarinic receptor subtype modulates vagally stimulated bronchial contraction. *J Appl Physiol* **65:**2144–2150.

Caulfield MP (1993) Muscarinic receptors—characterization, coupling and function. *Pharmacol Ther* **58:**319–379.
Caulfield MP and Birdsall NJM (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50:**279–290.
Escolar JD, Escolar MA, Guzman J, and Roques M (2003) Morphological hysteresis of the small airways. *Histol Histopathol* **18:**19–26.
Felder CC (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J* **9:**619–625.
Fisahn A, Yamada M, Duttaroy A, Gan JW, Deng CX, McBain CJ, and Wess J (2002) Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. *Neuron* **33:**615–624.
Fryer AD and el-Fakahany EE (1990) Identification of three muscarinic receptor subtypes in rat lung using binding studies with selective antagonists. *Life Sci* **47:**611–618.
Fryer AD and Jacoby DB (1998) Muscarinic receptors and control of airway smooth muscle. *Am J Respir Crit Care Med* **158:**154–160.
Garssen J, Van Loveren H, Gierveld CM, Van der Vliet H, and Nijkamp FP (1993) Functional characterization of muscarinic receptors in murine airways. *Br J Pharmacol* **109:**53–60.
Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, Brodkin J, Grinberg A, Sheng H, and Wess J (1999) Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci USA* **96:**1692–1697.
Hislop AA, Mak JC, Reader JA, Barnes PJ, and Haworth SG (1998) Muscarinic receptor subtypes in the porcine lung during postnatal development. *Eur J Pharmacol* **359:**211–221.
Jacoby DB and Fryer AD (2001) Anticholinergic therapy for airway diseases. *Life Sci* **68:**2565–2572.
Kilbinger H, von Bardeleben RS, Siefken H, and Wolf D (1995) Prejunctional muscarinic receptors regulating neurotransmitter release in airways. *Life Sci* **56:**981–987.
Lazareno S, Buckley NJ, and Roberts FF (1990) Characterization of muscarinic M4 binding sites in rabbit lung, chicken heart and NG108-15 cells. *Mol Pharmacol* **38:**805–815.
Kotlikoff MI, Kume H, and Tomasic M (1992) Muscarinic regulation of membrane ion channels in airway smooth muscle cells. *Biochem Pharmacol* **43:**5–10.
Maclagan J, Fryer AD, and Faulkner D (1989) Identification of M1 muscarinic receptors in pulmonary sympathetic nerves in the guinea-pig by use of pirenzepine. *Br J Pharmacol* **97:**499–505.
Mak JC, Baraniuk JN, and Barnes PJ (1992) Localization of muscarinic receptor subtype mRNAs in human lung. *Am J Respir Cell Mol Biol* **7:**344–348.
Martin C, Uhlig S, and Ullrich V (1996) Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. *Eur Respir J* **9:**2479–2487.
Martin RJ (2002) Therapeutic significance of distal airway inflammation in asthma. *J Allergy Clin Immunol* 109:S447–S460.
Matsui M, Griffin MT, Shehnaz D, Taketo MM, and Ehlert FJ (2003) Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contractions in smooth muscle from M2 receptor knockout mice. *J Pharmacol Exp Ther* **305:**106–113.
Matsui M, Motomura D, Fujikawa T, Jiang J, Takahashi S, Manabe T, and Taketo MM (2002) Mice lacking M2 and M3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. *J Neurosci* **22:**10627–10632.
Miyakawa T, Yamada M, Duttaroy A, and Wess J (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. *J Neurosci* **21:**5239–5250.
Reinheimer T, Mohlig T, Zimmermann S, Hohle KD, and Wessler I (2000) Muscarinic control of histamine release from airways. Inhibitory M1-receptors in human bronchi but absence in rat trachea. *Am J Respir Crit Care Med* **162:**534–538.
Roffel AF, Elzinga CR, and Zaagsma J (1990) Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm Pharmacol* **3:**47–51.
Shapiro MS, Gomeza J, Hamilton SE, Hille B, Loose MD, Nathanson NM, Roche JP, and Wess J (2001) Identification of subtypes of muscarinic receptors that regulate Ca^{2+} and K^{+} channel activity in sympathetic neurons. *Life Sci* **68:**2481–2487.
Spicuzza L, Basile L, Belvisi MG, Bellofiore S, Matera MG, Cazzola M, and Di Maria GU (2002) The protective role of epithelium-derived nitric oxide in isolated bovine trachea. *Pulm Pharmacol Ther* **15:**357–362.
Stengel PW, Gomeza J, Wess J, and Cohen ML (2000) M_2 and M_4 receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. *J Pharmacol Exp Ther* **292:**877–885.
Stengel PW, Yamada M, Wess J, and Cohen ML (2002) M_3-receptor knockout mice: muscarinic receptor function in atria, stomach fundus, urinary bladder and trachea. *Am J Physiol* 282:R1443–R1449.
Tilley SL, Hartney JM, Erikson CJ, Jania C, Nguyen M, Stock J, McNeisch J, Valancius C, Panettieri RA Jr, Penn RB, et al. (2003) Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. *Am J Physiol* **284:**L599–L606.
Watson N and Eglen RM (1999) Muscarinic receptor antagonists. *Pulm Pharmacol Ther* **12:**115–118.
Wohlsen A, Uhlig S, and Martin C (2001) Immediate allergic response in small airways. *Am J Respir Crit Care Med* **163:**1462–1469.
Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, Ogawa M, Chou CJ, Xia B, Crawley JN, et al. (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature (Lond)* **410:**207–212.
Zaagsma J, Roffel AF, and Meurs H (1997) Muscarinic control of airway function. *Life Sci* **60:**1061–1068.
Zhang W, Yamada M, Gomeza J, Basile AS, and Wess J (2002) Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M_1-M_5 muscarinic receptor knock-out mice. *J Neurosci* **22:**6347–6352.

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