J. vet. Pharmacol. Therap. **30,** 401–409.

Equine recurrent airway obstruction does not alter airway muscarinic acetylcholine receptor expression and subtype distribution

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Abraham, G., Kottke, C., Ungemach, F. R. Equine recurrent airway obstruction does not alter airway muscarinic acetylcholine receptor expression and subtype distribution. *J. vet. Pharmacol. Therap.* **30**, 401–409.

In recurrent airway obstruction (RAO) or heaves, bronchospasm has been attributed to enhanced cholinergic activity. However, the expression and function of muscarinic acetylcholine receptors (mAChR) and their signaling components are not yet known. Thus, we examined the expression, subtype distribution and postreceptor signaling pathways of mAChR in the peripheral lung, bronchial and tracheal epithelia with the underlying smooth muscle from nine horses with RAO and 11 healthy control horses. In RAO horses, no significant segment-dependent alteration in mAChR density and subtype distribution (assessed by [*N*-methyl-^{3}H]-scopolamine binding; ([^{3}H]-NMS)), was found, except a trend in receptor down-regulation in some peripheral parts of the lung. The total number of high mAChR binding sites (assessed by carbachol-displacement experiments in the presence or absence of guanosine 5′-triphosphate) was not changed in RAO, suggesting that the functional coupling of mAChR to the corresponding G-proteins is intact. The M_2-mediated inhibition of adenylate cyclase (AC) as well as the M_3-receptor-$G_{q/11}$-phospholipase C (PLC) activity was not different between RAO and control airway tissues. In conclusion, in equine RAO airways, mAChR expression and function were not altered, and thus appear not to account for the enhanced cholinergic activity in RAO.

(Paper received 19 June 2007; accepted for publication 24 July 2007)

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INTRODUCTION

Bronchial hyper-responsiveness, often associated with airway inflammation and infiltration of inflammatory cells (neurophils, eosinophils, mast cells and lymphocytes) (Laitinen *et al.*, 1985; Djukanovic *et al.*, 1990), is characterized by exaggerated bronchoconstrictory responses to airway constrictor substances (agonists). Although a number of hypotheses have been proposed, the underlying mechanisms responsible for such airway obstructions are still a matter of debate. In asthmatic human patients or model animals, increasing physiological and pharmacological evidence suggests that the autonomic innervation that controls the airway smooth muscle tone might be abnormal, and that an imbalance between the excitatory parasympathetic system via muscarinic acetylcholine receptors (mAChR) and the inhibitory sympathetic system via β-adrenergic receptors (β-ARs) might contribute to the genesis and/or manifestation of obstructive pulmonary diseases (Barnes, 1986; Insel & Wasserman, 1990).

Already Szentivanyi (1968) hypothesized that the function of the β-AR system is impaired and might constitute a potential cause of increased airway smooth muscle sensitivity in asthma. Moreover, impaired β-AR responsiveness has been shown in cholinergically stimulated airway smooth muscle from severe asthmatic patients (Goldie *et al.*, 1986; Bai, 1991; Bai *et al.*, 1992) and also from animal models of asthma (Rubinfeld *et al.*, 1982; Taki *et al.*, 1986; Wills-Karp & Gilmour, 1993; Hakonarson *et al.*, 1995). Indeed, dysfunction of the β-AR system has rarely been attributed to a decreased β-AR density or affinity in asthmatic airways (Nadel & Barnes, 1984; Spina *et al.*, 1989; Bai *et al.*, 1992; Haddad *et al.*, 1996). Nevertheless, we have recently shown that in equine recurrent airway obstruction (RAO), which resembles human asthma, the β-AR-G_s-protein-adenylate cyclase system is strongly impaired (Abraham *et al.*,

2007). In human asthmatic airways, β-adrenergic-mediated hypo-responsiveness seems to result from enhanced parasympathetic (cholinergic) activity, which was the rational for the therapeutic use of vagolytic agents (e.g. ipratropium bromide) as well as β_2-agonists (Belmonte, 2005). Although the reasons behind these defective mechanisms remain to be elucidated, it was assumed that changes in the expression of mAChR might occur in obstructive airway diseases depending on how frequently bronchial hyper-reactivity is observed (Coulson & Fryer, 2003). For example, in the human asthmatic lungs, neither alterations (Haddad *et al.*, 1996), nor an increase (van Koppen *et al.*, 1989) in total number of postsynaptic mAChR was observed. Accordingly, in asthmatic patients an increase in cholinergic contraction was associated with enhanced M_3-receptor function (de Jongste *et al.*, 1987a; Bai, 1990), but in various patients with chronic obstructive pulmonary diseases (COPD) no change was observed (de Jongste *et al.*, 1987b; van Koppen *et al.*, 1988). In contrast, a dysfunction of prejunctional M_2-receptors (autoreceptors), which control acetylcholine release via a negative feedback mechanism, appeared to occur in human asthma (Minette *et al.*, 1989) and also in asthma models in artificially sensitized animals (ten Berge *et al.*, 1996). In the latter case, however, no alteration in postsynaptic mAChR expression was identified (Whicker *et al.*, 1991; Lee *et al.*, 1994).

In horses with clinical signs of RAO, the contribution of mAChR to the disease pathogenesis has not been studied in detail, although *in vivo* as well as *in vitro* an increased pulmonary resistance was observed after atropine administration (Broadstone *et al.*, 1988; Wang *et al.*, 1995). Recently, we have characterized these cholinergic receptors in healthy horses. Results showed that three subtypes are predominant in the equine airways. Analysis of quantitative expression allowed the following overall ranking: $M_2>>M_3>M_1$, but receptor distribution was clearly segment dependent (Abraham *et al.*, 2007). In the present study, we examined the hypothesis that mAChR and postreceptor transmembrane signaling mechanisms might contribute to the mechanisms of obstructive pulmonary diseases in the horse. The density and distribution of mAChR as well as the corresponding effectors (G-protein coupling efficiency, adenylate cyclase, and phospholipase C activity) were investigated in the peripheral lung, bronchus and trachea of equine RAO patients.

MATERIALS AND METHODS

Chemicals

[*N*-methyl-^{3}H]-scopolamine chloride ([^{3}H]-NMS) (specific activity: 81 Ci/mmol), [α-^{32}P]-ATP (specific activity: 30 Ci/mmol) and [^{3}H]-PIP_2 (specific activity: 6 Ci/mmol) were purchased from Perkin–Elmer Life Sciences (Boston, MA, USA). [^{3}H]-cyclic AMP (specific activity: 42 Ci/mmol) was obtained from Amersham Biosciences. Muscarinic receptor antagonists, carbachol, (-)-isoproterenol, guanosine 5′-triphosphate (GTP), NaF, ATP and bovine serum albumin were from Sigma (St Louis, MO, USA). Cyclic adenosine 3′, 5′-monophosphate (cAMP), adenosine deaminase (ADA), phosphocreatine and creatine kinase were bought from Roche (Mannheim, Germany). All other chemicals were of analytical grade commercially available.

Tissue sampling

Control animals and horses with RAO were tissue donors for the receptor analysis *ex vivo*. The control group consisted of 11 horses of either sex and various breeds (average age 12 ± 1.3 years), free of signs of respiratory disease, as examined before slaughter. The RAO group consisted of nine horses (both sex and various breeds with an average age of 16 ± 2.1 years) with a history and significant signs of obstructive airway disease for a long time. Horses in both groups had not previously received any medications at least 3 months prior slaughter except routine vaccination.

Whole lobes of the lung and trachea were obtained immediately after slaughter. Gross inspections of the lungs revealed that in animals with RAO, lungs had emphysema and fibrosis, peribronchial swelling and mucus accumulation in the airways. The disease status was confirmed by histopathological investigations. Smooth muscle tissue, including epithelium, was isolated from the trachea, and secondary and small bronchi by removing connective tissue and blood vessels. Tissue samples (about 1 g) from each respiratory segment (lung, bronchi, trachea), were snap frozen in liquid nitrogen and stored at −70 °C until use.

Preparation of airway tissue membranes

Membranes were prepared from frozen peripheral lung sections, and secondary and small bronchi, as well as from tracheal epithelium with the underlying smooth muscle. Briefly, tissues were thawed on ice in 20 volumes (w/v) of ice-cold lysis buffer (20 mM $NaHCO_3$), minced with scissors and homogenized twice, each for 30 sec, at 10 000 ***g*** in 1-min intervals with an Ultra Turrax tissue homogenizer (Janke & Kunkel, Staufen, Germany). Homogenates were centrifuged at 500 ***g*** and 4 °C for 10 min, and the supernatant passed through four layers of cheese cloth and re-centrifuged at 20 000 ***g*** (Beckman Ultra-Centrifuge; Fullerton, CA, USA) for 30 min at 4 °C. After discarding the supernatant, pellets were resuspended in 15 mL lysis buffer and centrifuged again as above. For the determination of mAChR, the final pellets were resuspended in the incubation buffer (containing 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, and pH 7.4) to yield a final protein concentration of 1–2 mg protein/mL.

For the determination of adenylate cyclase activity final pellets were resuspended in 20 mM Tris–HCl buffer, pH 7.4, 25 °C, containing 1 mM EDTA and 25 mM NaCl.

Both membrane preparations were snap frozen in liquid nitrogen and stored at −70 °C until use. Protein concentration was determined according to the method of Lowry *et al.* (1951) (assessed with Folin reagent) using bovine serum albumin (Sigma, Deisenhofen, Germany) as the standard.

Muscarinic receptor binding assay

To identify whether the maximal mAChR binding sites (B_{max}) and the apparent affinity dissociation constant (K_D) were altered in animals with RAO, saturation binding studies have been carried out in membranes from the three tissues as recently detailed elsewhere (Abraham *et al.*, 2007). Briefly, tissue membranes (150 μL with 100–300 μg protein) were incubated with increasing concentrations of [^{3}H]-NMS ranging from 0.1 to 10 nM in a final volume of 250 μL incubation buffer (see above). Incubations were carried out at 25 °C for 60 min and terminated by rapid filtration of the reaction mixture over GF-52 glass fiber filters (Schleicher and Schuell, Dassel, Germany) using a 1225-sampling manifold (Millipore Corp., Schwalbach, Germany). After washing and subsequently drying of each filter, the radioactivity was measured using a Wallac Scintillation Counter (Perkin–Elmer Life Sciences) with a counting efficiency of ~50%. Specific binding was defined as the difference between total [^{3}H]-NMS binding in the absence, and nonspecific binding in the presence of the nonselective muscarinic receptor antagonist atropine (1 μM). Specific binding was 80–90% (at 0.1–3 nM) and 70% (at 10 nM) of [^{3}H]-NMS, in the investigated samples, except for peripheral lung tissue membranes.

Competition binding assays

Muscarinic receptor subtypes were determined by [^{3}H]-NMS competitive displacement studies. An aliquot of membrane samples from each tissue was incubated with 2–3 nM [^{3}H]-NMS in the presence or absence of increasing concentrations (0.1 nM–100 μM) of subtype-selective muscarinic receptor antagonists in the incubation buffer. Telenzepine was used to identify M_1-subtype binding sites; methoctramine to characterize M_2 binding sites, although it is known to have additional M_4 activity, and 4-DAMP, which preferentially blocks M_3-subtypes, but also M_1, M_4 or M_5 subtypes with low affinity.

G-protein coupling to mAChR

To test whether the signal transduction pathway via G-protein coupling to the corresponding receptor might be altered in RAO patients, we determined the number of receptors in the high- and low-affinity state in membranes from three tissues, and assayed GTP effects on the inhibition of [^{3}H]-NMS binding by the muscarinic agonist carbachol. Briefly, membranes were pretreated either with vehicle or with GTP (100 μM), and carbachol at increasing concentrations (1 nM to 100 μM final concentration) was used to compete with [^{3}H]-NMS (2–3 nM) binding. The amount of specific [^{3}H]-NMS binding was determined as described above.

Adenylate cyclase activity

As the M_2-subtype of mAChR predominates in the airways, we evaluated whether or not the mAChR-mediated cAMP formation via G_i-protein stimulation was altered in tissues of RAO patients, and the adenylate cyclase (AC) activity was determined as recently described in detail (Abraham *et al.*, 2003). To investigate whether increases in the AC activity by stimulation of β-adrenoceptor and G_S-protein can be inhibited via the M_2-acetylcholine receptor subtype, we examined the inhibitory effect of carbachol (either concentration-dependently or with a fixed concentration of 1 mM).

Phospholipase C assay

For the determination of phospholipase C (PLC) activity, crude membranes of each tissue were prepared by repeated centrifugation and homogenization in hypotonic Tris/EGTA buffer (20 mmol/L Tris–HCl, 5 mmol/L EGTA, pH 7.2) as described above. The pellets obtained were then resuspended at a protein concentration of 2.5 mg/mL in cold incubation buffer containing 25 mmol/L Tris–Base, 8 mM LiCl, 6 mM $MgCl_2$, 3 mM ATP, 1 mM sodium deoxycholate, pH 6.8. The assay was carried out according to the method described by Garro *et al.* (2001). In brief, 40 μL membrane suspension (60–100 μg protein/tube) was incubated with [^{3}H]-PIP_2 (10 000–15 000 cpm) and incubation buffer additionally containing $CaCl_2$ necessary to yield 50 nmol/L free calcium, GTPγS (5 μmol/L), 1 mmol/L carbachol in the presence of GTPγS (5 μM) and NaF (10 mM), at a total volume of 100 μL. Tubes were incubated at 37 °C for 15 min. The reaction was stopped by adding 1.2 mL ice-cold chloroform/methanol (1:2 v/v). Thereafter, chloroform and 0.25 M HCl (each 0.5 mL) were added to each sample. After vigorously mixing, samples were kept on ice for 20 min to establish two phases, and then centrifuged at 1000 ***g*** for 30 sec. 1 mL aliquots of the upper aqueous phases containing [^{3}H]-inositol phosphates was mixed with 3 mL of Rotizint® 22 for scintillation counting.

Data analysis and statistics

Results in the text, tables and figures are given as mean values ± SEM of the number of animals tested. The receptor density (B_{max}) and the dissociation constant (K_D) values were analyzed from each animal and were averaged to permit statistical comparisons (GraphPad Software, San Diego, CA, USA). The IC_{50} values for antagonist and agonist displacement of [^{3}H]-NMS binding were calculated from concentration inhibition curves and transformed to K_I values using the approximation: $K_I = IC_{50}/[L]/K_D + 1$, where IC_{50} is defined as the concentration of competing agonists and antagonists required to inhibit 50% of the specific [^{3}H]-NMS binding and [L] is the concentration of [^{3}H]-NMS in the assay. For statistical analysis the F-ratio test was carried out to determine the goodness of fit of the concentration inhibition curves for either one- or two-site competition for [^{3}H]-NMS binding sites. The F-test compares the fit of two equations, where the more complicated equation (the one with more parameters) fits better (has a smaller sum-of-squares) than the simple equation (GraphPad Software).

Statistical significance of differences between control and RAO horses was estimated by the nonpaired two-tailed student's

t-test. All statistical calculations were performed with GraphPad Software. The level of significance was set at $P < 0.05$.

RESULTS

Pulmonary muscarinic acetylcholine receptor density

In all three tissues (lung, bronchi and trachea) of non-RAO and RAO-positive animals, the specific [^{3}H]-NMS binding reached saturation with increasing concentrations of free [^{3}H]-NMS revealing hyperbolic curves between 3 and 10 nM. In membranes of the trachea of healthy control animals, the extrapolated number of maximal binding sites (B_{max}) was significantly higher when compared with bronchus (1.6-fold) and peripheral lung (32-fold), respectively ($P < 0.01$; Table 1 and Fig. 1). In RAO lung membranes, the maximal density of [^{3}H]-NMS binding sites was reduced, but did not reach statistical significance ($P = 0.053$ vs. control, Fig. 1 and Table 1). RAO did not cause an alteration in mAChR density in bronchial and tracheal membranes (Fig. 1 and Table 1). The receptor affinities (K_D values) for [^{3}H]-NMS in RAO were not statistically different from those in healthy animals (Table 1).

Table 1. Muscarinic acetylcholine receptor density (B_{max}) and affinity for [^{3}H]-NMS (K_D) in the lung, bronchial and tracheal membranes

	n	Lung	Bronchus	Trachea
B_{max} (fmol/mg protein)				
Control	11	22 ± 3	438 ± 48	720 ± 60
RAO	9	15 ± 2	407 ± 64	752 ± 49
K_D (nM)				
Control	11	1.6 ± 0.7	1.9 ± 0.3	1.7 ± 0.2
RAO	9	1.6 ± 0.3	1.5 ± 0.2	1.4 ± 0.1

Data were obtained from saturation [^{3}H]-NMS binding studies, and are given as mean ± SEM values of *n* experiments.

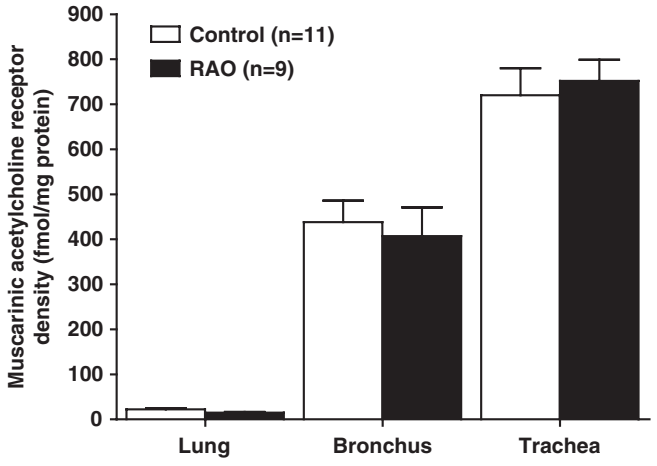

Fig. 1. Total mAChR density in control (open bars) and RAO (closed bars) tissues. Data shown are mean ± SEM values of 11 (control) and nine (RAO) experiments, each performed in duplicate.

Regional comparison of muscarinic acetylcholine receptor subtypes

As demonstrated in Fig. 2, the ratio of mAChR subtype distribution in the three tissues was also not significantly

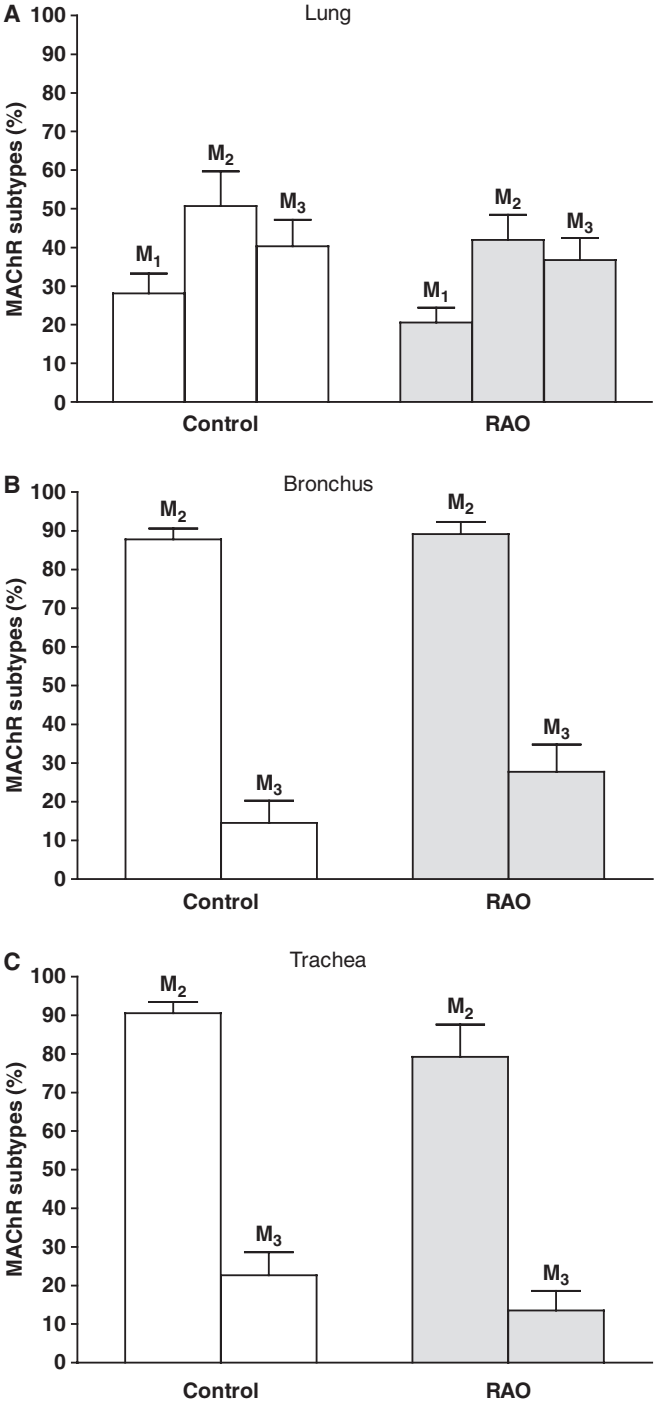

Fig. 2. Muscarinic acetylcholine receptor distribution in membranes of peripheral lung, bronchus and trachea of control (open bars) and RAO (closed bars) animals. Bar graphs show the percentage of mAChR subtypes distribution in control and RAO tissues. Data were mean ± SEM values of duplicate determinations from 11 healthy and nine RAO horses.

different between RAO and healthy horses. In the lung of control animals M_1:M_2:M_3 ratio was 28%:51%:40% and in RAO lung 21%:42%:37%. The M_2:M_3 ratio in the bronchus of controls (87%:14%) was also similar to RAO horses (89%:27%), and the tracheal M_2:M_3 ratio in controls (90%:22%) did not differ from RAO (79%:14%). In the latter two tissues, only M_2 and M_3 are expressed, with the former representing 70–90% of the total mAChR and the remaining receptors being of the M_3-AChR subtype (~4:1 ratio).

G-protein coupling to muscarinic acetylcholine receptors

In the three examined control tissues, about 35% of mAChR was found in state of high-affinity binding, i.e. receptor binding sites which functionally couple to their respective G-protein (Abraham *et al.*, 2007). In all three tissue membranes of healthy and RAO animals, nonlinear regression analysis of the concentration-inhibition curves of carbachol demonstrated a graphical presentation which fitted better to a two-site binding than one-site binding model (F-ratio test, $P < 0.01$; Table 2). Neither the percentage of the mAChR at high-affinity state (31–37%) nor the K_I-value at the high-affinity state (7 nM) was affected by RAO; the same was true for K_I-values at low-affinity states (4.3–4.8 μM) (Fig. 3a–c and Table 2). In tissue membranes of both animal groups, the addition of 100 μM GTP to carbachol competition binding assays shifted the concentration inhibition curves to the right, to the low-affinity state in a similar manner (K_I value: ~ 5 μM, Table 2), which fitted better to an one-site than a two-site binding model (Fig. 3).

Adenylate cyclase activity

M_2-receptor subtype-mediated inhibition of AC (and synthesis of cAMP) was compared between RAO and control airway tissues. Muscarinic and β-adrenergic receptor/G-protein coupling to AC were determined in the presence of carbachol and isoproterenol plus GTP. Both in control and RAO animal tissue membranes, the increase in GTP plus isoproterenol-stimulated AC activity was equally attenuated by carbachol (Fig. 4).

Phospholipase C activity

We finally assessed the activity of PLC. GTPγS alone or in combination with carbachol stimulated the hydrolysis of [^{3}H]-PIP_2 above basal levels, but there was no statistical difference between both groups (Table 3). In addition, NaF, which activates all G-proteins, had no statistically different effect on [^{3}H]-PIP_2 hydrolysis between examined control and RAO airway tissues.

DISCUSSION

A common problem in horses with RAO and human patients with asthma is the development of an autonomic dysfunction (Jartti, 2001; Matera *et al.*, 2002), including decreased responsiveness of the sympathetic β-adrenergic mechanisms and increased parasympathetic cholinergic activity with enhanced bronchospasm (Barnes, 1986). In RAO horses, we have recently shown that the airway β-adrenergic receptor-G_s-protein-adenylate cyclase system is impaired (Abraham *et al.*, 2007), whereas relatively little is known about pulmonary mAChR in RAO horses. So far, no data were available; we investigated the expression and signal-transduction pathways of these receptors in RAO airways. We hypothesized that the exaggerated bronchoconstriction (bronchospasm) which occurs during RAO might result from (1) high expression of the postsynaptic M_3-receptors which in turn via activation of $G_{q/11}$-protein and PLC increase the intracellular Ca^{2+} concentration, and thus, the contractile responses of the airway smooth muscle; and/or (2) increased numbers of postsynaptic M_2-receptors which functionally antagonize the β-AR function and similarly induce contraction by inhibiting the adenylate cyclase via G_i-protein activation (Emala *et al.*, 1995).

Generally, in the equine airways, there was a clear segment-dependent expression and subtype distribution of mAChR (Abraham *et al.*, 2007). The mAChR density was higher in the trachea followed by the bronchus and peripheral lung, whereby the M_2- and M_3-subtypes predominated, with the former representing 50–90% and the latter 30–40%, in accordance with data obtained from airways of several mammalian species

Table 2. High- and low-affinity binding properties of carbachol

	Control			RAO		
	K_{IH} (nM)	K_{IL} (μM)	R_H (%)	K_{IH} (nM)	K_{IL} (μM)	R_H (%)
Carbachol						
Lung	7.6 ± 0.2	4.7 ± 0.2	37 ± 4	7.4 ± 0.2	4.8 ± 0.2	36 ± 4
Bronchus	7.2 ± 0.3	5.1 ± 0.2	31 ± 3	7.5 ± 0.3	4.6 ± 0.1	32 ± 3
Trachea	6.6 ± 0.1	4.4 ± 0.1	33 ± 3	6.8 ± 0.2	4.3 ± 0.1	37 ± 2
Carbachol + 100 μM GTP						
Lung		5.1 ± 0.2			5.2 ± 0.1	
Bronchus		4.7 ± 0.1			4.8 ± 0.1	
Trachea		4.7 ± 0.1			4.8 ± 0.1	

Data presented are mean ± SEM values of 11 (control) and nine (RAO) horses performed in duplicate. K_{IH} and K_{IL} are high- and low-affinity constants for carbachol, respectively, obtained in the absence of GTP. R_H (%) is the percentage of receptors in the high-affinity state.

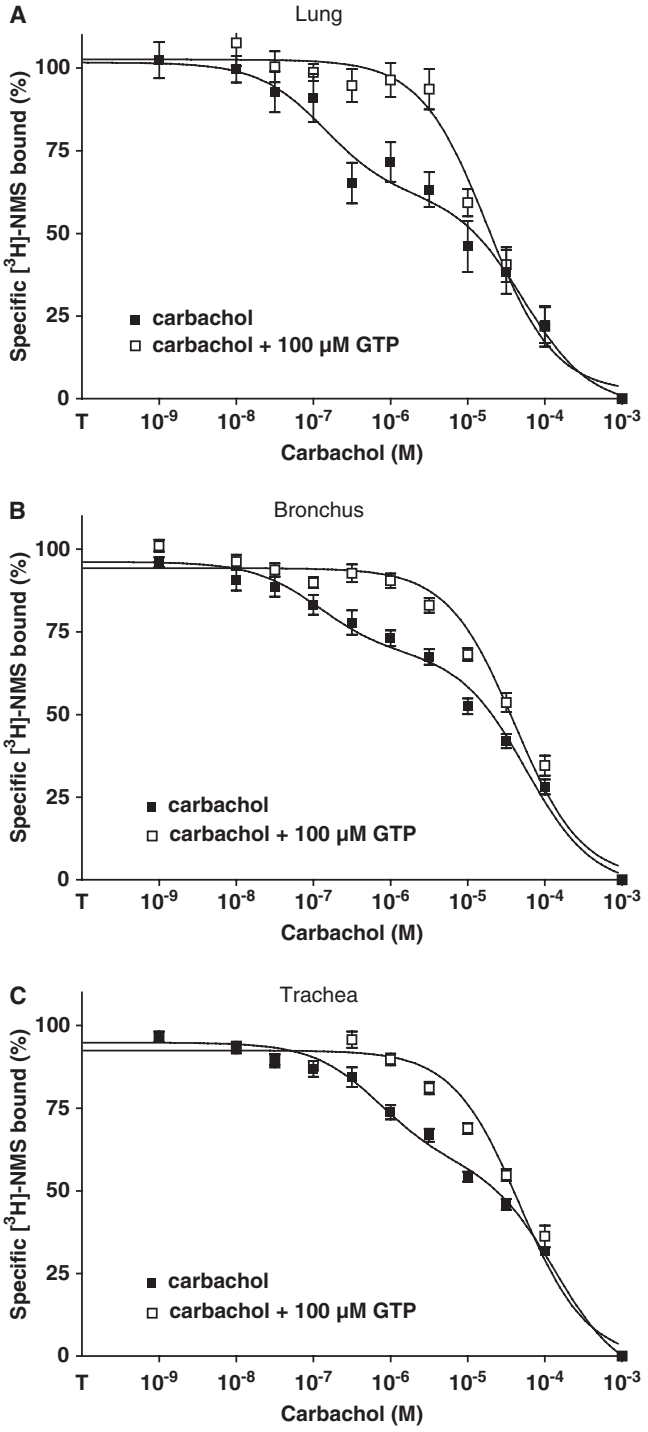

Fig. 3. Effect of GTP on carbachol concentration-inhibition curves in RAO lung, bronchial and tracheal membranes. 100% binding represents the specific [^{3}H]-NMS binding detected with 1 μM atropine in the absence of carbachol. Data were mean ± SEM values of duplicate determinations from nine RAO horses.

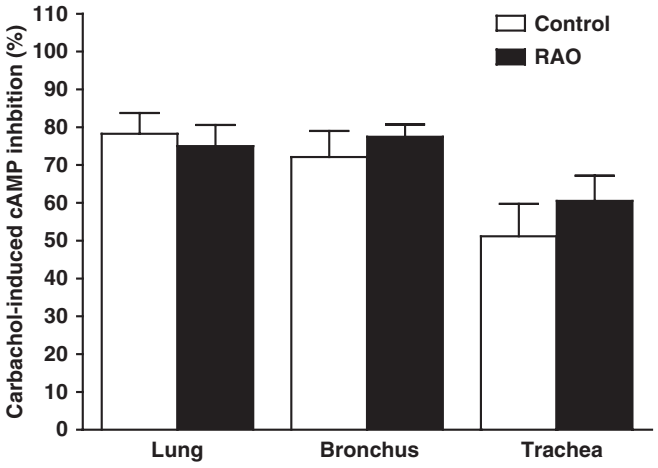

Fig. 4. Activation of adenylate cyclase in airway tissue membranes from control and RAO animals. Ordinates: mean net increase in pulmonary adenylate cyclase activity expressed as cAMP formation in percentages. The response was measured under basal conditions and in the presence of 10 μM GTP plus 10 μM isoproterenol. GTP plus isoproterenol-stimulated adenylate cyclase activity was inhibited by carbachol and showed no statistical difference between control and RAO tissues.

Table 3. Stimulation of [^{3}H]-PIP_2 hydrolysis in control and RAO airway tissue membranes ($n = 6$)

	Lung	Bronchus	Trachea
GTPγS			
Control	5.8 ± 1.1	9.5 ± 1.6	6.4 ± 1.4
COB	8.1 ± 3.1	8.2 ± 1.3	8.6 ± 2.4
Carbachol + GTPγS			
Control	12.4 ± 3.5	12.5 ± 2.5	11.2 ± 3.0
COB	15.2 ± 5.9	12.4 ± 2.9	11.4 ± 2.1
Sodium fluoride			
Control	15.1 ± 2.7	16.9 ± 2.4	19.4 ± 3.4
COB	14.8 ± 4.0	17.7 ± 1.4	19.3 ± 4.0

Values are expressed as fmol hydrolyzed PIP_2/mg protein/min.
Data were presented after subtraction of the basal values, and represent mean ± SEM values of six experiments.

including humans (Roffel *et al.*, 1988; Lucchesi *et al.*, 1990; Haddad *et al.*, 1994). In the equine RAO airways, our data indicate that there was no statistically significant difference in receptor density and subtype distribution when compared with control airway tissues. Only a trend for measurable alteration in mAChR concentration has been observed in the peripheral lung, but this did not reach statistical significance. These observations in RAO airways were in accordance with the data found in asthmatic airways in humans, indicating nonsignificant changes in mAChR density and receptor mRNA expression (Haddad *et al.*, 1996). The decreased number of mAChR in the RAO peripheral lung was in part in line with that reported by Raaijmakers *et al.* (1989) in the peripheral lung from human patients with COPD. However, in the latter case, there was a statistically significant diminution (~70%) of the mAChR density. On the other hand, our present data contrast the slight but not significant increase (8–25%) in total number of mAChR in central and segmental airway smooth muscle of patients suffering from chronic and severe airflow obstruction (van Koppen *et al.*, 1989). Similarly, one research group has demonstrated increased expression of the

mAChR in the tracheal smooth muscle of Basenji-greyhound dogs with nonspecific airway hyper-responsiveness (Emala *et al.*, 1995).

The discrepancies discussed above suggest that in obstructive airway diseases, changes in mAChR either do not occur at all, or might be related to the disease severity or to specific receptor subtypes and their functional responses. The latter hypothesis was not supported in human COPD patients, as the agonist-induced cholinergic activity of isolated airway smooth muscle was not increased (de Jongste *et al.*, 1987a; van Koppen *et al.*, 1988), indicating that M_3-subtype function may not be enhanced in obstructive airway diseases. In Basenji-greyhound dogs, however, an enhanced cholinergic activity could partially be attributed to an increased number of M_2-receptors (Emala *et al.*, 1995) which functionally antagonize the β_2-adrenergic receptors. In addition, in ovalbumin sensitized guinea-pig airways, increased expressions of the M_3-subtype (Tohda *et al.*, 2002) has been associated with increased cholinergic activity, which would directly induce smooth muscle contractile responses. Moreover, in RAO horses, *in vitro* functional studies did not provide consistent data with regard to disease-related dysfunction of mAChR. For example, acetylcholine did not increase contraction of isolated RAO airway smooth muscle (Broadstone *et al.*, 1991; LeBlanc *et al.*, 1991), suggesting unchanged mAChR function in RAO, in agreement with our present data. On the other hand, electrical field stimulation of airway smooth muscle increased contractile responses, suggesting increased acetylcholine releases and a defect in mAChR function (Zhang *et al.*, 1999). The inconsistent results from the literature as to whether in stable human asthma or RAO the mAChR density is altered or unchanged may not clearly explain the site of functional defect in mAChR signaling processes.

To the best of our knowledge, this is the first time that consideration has been given as to whether the coupling of mAChR to the corresponding effector proteins and respective G-proteins might be altered in RAO. In the equine airways, M_2- and M_3-receptors predominate, the former which couple to G_i-proteins and inhibit AC (hence cAMP formation) and the latter which couple to $G_{q/11}$-proteins and activate PLC to generate IP_3 and DAG, thus both antagonize signaling pathways that mediate β_2-receptor-dependent smooth muscle relaxation. When comparing the number of high-affinity binding sites of mAChR between RAO and control airways tissues, there was no statistical difference (in both cases 31–37%), thus, indicating unaltered coupling of the signal-transducing protein to mAChR in RAO. In tissues from asthmatic human patients, no known study investigated the effects on the interaction of the mAChR with G-proteins. However, in some animal asthma models, the expression of the G_i-protein was enhanced, which does not generally mean that coupling efficiency is also enhanced (Hakonarson *et al.*, 1995). Furthermore, the carbachol-induced inhibition (via M_2-receptor) of isoproterenol- plus GTP-stimulated AC activity was similar between RAO and control airway tissues, as well as the $G_{q/11}$-protein-mediated activation of PLC (via the M_3-receptors). The unchanged AC activity in RAO airways contrasts the reports by Emala *et al.* (1995) and Hakonarson *et al.* (1995), demonstrating increased inhibition of AC in airways of animal asthma models.

Regarding the underlying mechanisms of enhanced cholinergic activity in RAO, almost no studies have been carried out. Because we did not observe a difference in mAChR density and subtype distribution between horses with RAO and normal horses, it can be concluded that the function of these receptors might not be altered, thus this does not strictly support the ideas on the dysfunction of mAChR in asthma in humans or animal models (for a review see Coulson & Fryer, 2003; Belmonte, 2005). Moreover, the unchanged activity of AC and PLC for the corresponding mAChR subtypes in RAO, as well as the normal functional coupling to G-proteins, strongly supports the resistance of the mAChR signal-transducing mechanisms, which counteract the bronchodilation mediated by the β-adrenergic receptors (Abraham *et al.*, 2007). At present, the possible contribution of the mAChR and their signal transduction pathways to the enhanced bronchoconstriction in RAO cannot be confirmed (in the face of unchanged number and activity of G-proteins, AC and PLC). Thus, bronchospasm and/or enhanced parasympathetic activity might occur nonspecifically together with other spasmogenic factors and unchanged mAChR. Because of the study design, our data, however, do not allow to conclude in favor or against the involvement of mAChR in the pathogenesis of RAO in the horse, but strongly support the need for further investigation, e.g. with regard to cross-talk mechanisms between β-AR and mAChR mechanisms as well as functional studies. Moreover, further studies should examine the autonomic receptor mechanisms in RAO horses during acute exacerbation and long-term remission.

In summary, in RAO airways, mAChR number and subtypes, as well as the AC and PLC activity and their functional coupling to G-proteins, were not altered, in contrast to the impaired β-adrenergic receptor-G-Protein-AC-system in RAO. In RAO, mAChR may antagonize the function of the latter receptor mechanisms without increased numbers and/or enhanced signal-transduction via these receptors. It appears that these mechanisms, if indeed found to be important in RAO of horses, may provide the opportunity of using anticholinergic bronchodilators in combination with the standard β-adrenergic therapy regimen using long-acting β-adrenergic receptor agonists, e.g. clenbuterol.

ACKNOWLEDGMENTS

The skilful technical assistance of Martina Wieczorek and Ina Hochheim is gratefully acknowledged.

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