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Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts

S. Haag[1], S. Matthiesen[1], U.R. Juergens[2], K. Racké[1]

[1]**Institute of Pharmacology & Toxicology and**

[2]**Department of Pulmonary Diseases, Medical Polyclinic,**

University of Bonn, Germany

Short title:
Muscarinic stimulation of collagen synthesis

Correspondence:
Kurt Racké
Institute of Pharmacology & Toxicology
University of Bonn
Reuterstraße 2b
D-53113 Bonn
Germany
Email: racke.kurt@uni-bonn.de

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ABSTRACT

Clinical observations indicate that in COPD patients the long-acting muscarinic antagonist tiotropium delays decline in airway function suggesting that cholinergic mechanisms contribute to long-term structural changes. Human lung fibroblasts express muscarinic receptors and the present study aimed to explore their role in controlling collagen synthesis.

MRC-5, HEL-299 and primary human lung fibroblasts (phLFb) were cultured and incorporation of [^{3}H]-proline into cellular proteins was determined as measure of collagen synthesis.

In MRC-5 cells the muscarinic agonist carbachol enhanced [^{3}H]-proline incorporation in a concentration dependent manner (EC_{50}: 220 nM, increase at 10 µM by 40-55%, in different series of experiments). Likewise, 10 µM oxotremorine caused an increase by about 65%. For comparison, TGF-$ß_1$ (5 ng/ml) caused an increase by about 80%. Effects of carbachol on total [^{3}H]-proline incorporation and collagenase-sensitive [^{3}H]-proline fraction were similar. The effect of 10 µM carbachol was inhibited by tiotropium (IC_{50}: 110 pM), prevented by pertussis toxin and the MAP kinase inhibitor PD 98059. Muscarinic agonists enhanced [^{3}H]-proline incorporation in a tiotropium-sensitive manner also in HEL-299 cells and phLFb.

In human lung fibroblasts muscarinic receptors exert stimulatory effects on collagen synthesis. Prolonged blockade of muscarinic-induced collagen synthesis may contribute to reported long-term beneficial effects of anticholinergics in COPD.

Key words

Muscarinic receptors, tiotropium, collagen synthesis, lung fibroblasts, airway remodelling, MAP kinase

INTRODUCTION

Airway remodelling is a pathological feature observed in chronic inflammatory and obstructive airway diseases. Although airway remodelling processes in asthma and COPD are somewhat different, fibrotic alterations are observed in both diseases [1-3]. Based on their bronchodilatatory effects anticholinergic drugs constitute an essential element in the therapy of obstructive airway diseases, particularly in COPD [4]. Moreover, preliminary clinical data indicate that in patients with COPD, treatment with the long-acting muscarinic antagonist tiotropium may delay the decline in airway function [5,6], suggesting that cholinergic mechanisms may contribute to long-term structural changes, in addition to the well known acute bronchoconstrictory and secretory effects [7,8]. Indeed, muscarinic agonists were found to enhance the proliferative response of human and bovine airway smooth muscle to growth factors [9,10]. Moreover, in sensitized guinea pigs tiotropium attenuated airway smooth muscle mass thickening and prevented mucus gland hypertrophy induced by repeated allergen challenges [11,12].

Since fibrotic alterations are crucial in airway remodelling [1-3] we recently started to explore expression and function of muscarinic receptors in pulmonary fibroblasts. We revealed expression of different muscarinic receptors in primary human lung fibroblasts as well as in MRC-5 cells, a human fibroblast cell line derived from normal fetal lung tissue [13] and in both cell types M_2 appeared to be the predominant receptor subtype [14]. Moreover, in both cell types muscarinic agonists induced a proliferative response [14,15].

The present experiments aimed to characterize further the functional significance of muscarinic receptors expressed in human pulmonary fibroblasts and investigated whether collagen synthesis is controlled by muscarinic mechanisms.

MATERIALS AND METHODS

Culture of lung fibroblasts

MCR-5 human lung fibroblasts (CCL-171, ATCC, Manassas, USA) and HEL-299 (CCL 137, ATCC, Manassas, USA) were grown in Eagle's MEM supplemented with 10% FCS, 2 mM L-glutamine, Earle's BBS adjusted to contain 2.2 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified incubator at 37°C and 5% CO_2 and passaged by trypsinization at nearly confluence.

Primary human lung fibroblasts were established from normal areas of surgically resected lung tissue which was obtained from lung cancer patients after thoracotomy. Anonymous lung tissue of male Caucasians was obtained from patients who gave informed consent. The protocol for obtaining human tissue was approved by the local ethical review board for human studies (Ethics Committee, Medical Faculty, University of Bonn, Germany). Tissue was cut into small pieces, treated with pronase (1 mg/ml, Calbiochem Novabiochem, San Diego, CA, USA) at 37°C for 30 min, placed in cell culture plates and incubated in Eagle's MEM supplemented as described above, with FCS increased to 15 %. After 2 weeks fibroblasts had grown out from the tissue and were passaged by standard trypsinization. For experiments described here, cells of passage 3 - 11 were used.

Extraction of RNA and reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated by help of silica-gel-based membranes according to manufacturer's instructions including an additional DNase digestion protocol to beware any contamination by genomic DNA (Qiagen, Hilden, Germany). First strand cDNA was synthesised using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Specific oligonucleotide primers

were constructed based on human EMBL sequences: β-actin, 5′-CACTCTTCCAGCCTTCCTTC-3′ and 5′-CTCGTCATACTCCTGCTTGC-3′; M_1 receptor, 5′-CAGGCAACCTGCTGGTACTC-3′ and 5′-CGTGCTCGGTTCTCTGTCTC-3′; M_2 receptor, 5′-CTCCTCTAACAATAGCCTGG-3′ and 5′-GGCTCCTTCTTGTCCTTCTT-3′; M_3 receptor, 5′-GGACAGAGGCAGAGACAGAA-3′ and 5′-GAAGGACAGAGGTAGAGTGG-3′; M_4 receptor, 5′-ATCGCTATGAGACGGTGGAA-3′ and 5′-GTTGGACAGGAACTGGATGA-3′; M_5 receptor, 5′-ACCACAATGCAACCACCGTC-3′ and 5′-ACAGCGCAAGCAGGATCTGA-3′. PCR amplification was performed using Taq DNA polymerase in a programmable thermal reactor with initial heating for 3 min at 94°C, followed by 23 (ß-actin) respectively 35 (M_1-M_5) cycles of 45 s denaturation at 94°C, annealing at 53-60°C (30 s), extension at 72°C (1 min) and final extension for 10 min at 72°C. PCR products were separated by 1.2% agarose gel electrophoresis. Optical density of bands was quantified by RFLPscan 2.01 software (MWG, Ebersberg, Germany), corrected over ß-actin and referred to respective amplification of genomic DNA to normalize for variations in PCR effectiveness.

[^{3}H]-proline incorporation

Collagen synthesis and deposition into the extracellular matrix were assessed by [^{3}H]-proline incorporation assays originally developed by Peterkofsky and Diegelmann [16] and subsequently used in many studies [e.g. 17-20]. Cells were trypsinized, harvested and seeded into 12-well dishes at a density of 10^5 cells per well. Cells were first cultured for 24 h in presence of 10% FCS, followed by an additional 24 h under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 h. At the end, culture medium was removed and cells were washed twice with 4°C cold PBS followed by 1 h incubation in 1 ml 20% trichloric acid (TCA) at 4°C. Denaturated cells were scraped off, transferred into a reaction tube and centrifuged. The

pellet was washed with 1 ml 10% TCA and dissolved in 300 µl 0.2 M NaOH followed by neutralization with 300 µl 0.2 M HCl. 300 µl portions were combined with scintillation cocktail (PerkinElmer, Rodgau - Jügesheim, Germany) and radioactivity was determined by liquid scintillation spectrometry in a Packard 2100 TR liquid scintillation analyzer. External standardisation was used to correct for counting efficiency. Three wells of each 12-well culture dish served as controls and data are expressed as percentage of the mean of these individual controls of each cell preparation. In addition [^{3}H]-proline incorporation was also expressed in absolute terms (d.p.m).

In selected experiments 100 µl of collagenase II solution (2 mg/ml in TES buffer containing 50 mM Tris-Base, 5 mM EDTA, 20 mM NaCl, 5 mM $CaCl_2$) were added to 300 µl of neutralized protein extract followed by 1 h incubation at 37°C. The digestion was terminated by addition of 100 µl 50% TCA. Samples were centrifuged at 13,000 rpm for 10 min and radioactivity in the supernatant (as a measure of collagenase-sensitive [^{3}H]-proline) was determined by liquid scintillation counting. Releasable radioactivity was expressed as % of the total incorporated radioactivity in each individual experiment.

Statistical Analysis

Data are presented as scatter plots of the individual observations. In addition mean values (±S.E.M.) were calculated and it is referred to these values when drug effects are described quantitatively in the text. When normal distribution was confirmed by performing D'Agostino & Pearson omnibus normality test, statistical significance of differences was evaluated by ANOVA followed by Dunnett or Bonferroni test using GraphPad Prim5 (GraphPad Software, San Diego, USA). When normal distribution could not be confirmed (in all series with $n<8$,) significance of differences was evaluated by Kruskal-Wallis test followed by Dunn´s test. $P < 0.05$ was accepted as being significant. IC_{50} values were calculated by the use of computer programmes (GraphPad Prism5).

Drugs and Materials

Butaprost, carbachol (carbamylcholine chloride), nicotine bitatrate, oxotremorine sesquifumerate, PD-98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one], penicillin-streptomycin solution, pertussis toxin, transforming growth factor-ß$_1$ (TGF-ß$_1$) and trypsin were purchased from Sigma (Deisenhofen, Germany); collagenase II from CellSystems (St. Katharinen, Germany). Tiotropium bromide was a gift from Boehringer Ingelheim (Biberach, Germany). Eagle's minimal essential medium (MEM) with Earl's salts and L-glutamine, non-essential amino acids were from PAA (Cölbe, Germany), fetal calf serum (FCS) from Biochrom (Berlin, Germany), Taq DNA-polymerase from Invitrogen (Karlsruhe, Germany), Omniscript reverse transcriptase, RNeasy Mini kit and RNase-free DNase set from Qiagen (Hilden, Germany). Oligodesoxynucleotides for RT-PCR were obtained from MWG Biotech (Ebersberg, Germany)

RESULTS

Muscarinic receptor expression in human lung fibroblasts

In extension to the above mentioned previous observations an abundant expression of M_2 receptor mRNA was confirmed in a larger number of observations in primary human lung fibroblasts and MRC-5 cells and in addition also in HEL-299 cells, a further human lung fibroblast cell line [21] (Fig. 1). In all cells mRNA encoding M_2 receptor was by far the most prominent muscarinic receptor transcript. The levels of M_2 receptor mRNA appear to be quite similar in primary human lung fibroblasts and the two cell lines studied (Fig. 1A).There were some differences with regard to the expression of other muscarinic receptor subtypes. M_1 receptor transcript was only detected in primary cells whereas the levels of M_3 transcripts were highest in MRC-5 cells followed by HEL-299 cells and primary cells (Fig. 1B-D).

Muscarinic stimulation of collagen synthesis in MRC-5 human lung fibroblasts

Under control conditions, i.e. absence of FCS and any test drug, [^{3}H]-proline incorporation into cellular proteins amounted to 18,303 ± 981 d.p.m. (n=84), since there was no significant difference between the different series of experiments, a pooled value is given. The muscarinic receptor agonist carbachol enhanced [^{3}H]-proline incorporation in a concentration dependent manner, maximally by about 55% at 10 µM and with an EC_{50} of 220 nM (Fig. 2A). Likewise, 10 µM oxotremorine caused an increase by about 65% (Fig. 2A). For comparison, TGF-ß$_1$, a well known activator of fibroblast collagen synthesis caused an increase by about 85%, whereas the prostanoid EP_2 receptor agonist butaprost (100 nM) and forskolin (10 µM), a direct activator of adenylyl cyclase, inhibited [^{3}H]-proline incorporation by about 50% (Fig. 2B). About 80% of the [^{3}H]-proline was released from the protein fraction following 1 h digestion by collagenase II (Fig. 3A), confirming that total [^{3}H]-proline incorporation largely reflects specific collagen synthesis. Furthermore, the effects of carbachol and butaprost on collagen-sensitive [^{3}H]-proline were comparable to those on total [^{3}H]-proline incorporation (Fig. 3B).

The stimulatory effect of 10 µM carbachol on [^{3}H]-proline incorporation was inhibited in a concentration dependent manner by the muscarinic receptor antagonist tiotropium with an IC_{50} of 110 pM (Fig. 4). Pre-treatment of the cells with pertussis toxin tended to decrease (by about 20%) basal [^{3}H]-proline incorporation and prevented the stimulatory effect of 10 µM carbachol (Fig. 5A). Furthermore, PD 98059 (30 µM), a specific inhibitor of the MAPK-activating enzymes (MEK) [22] did not affect basal [^{3}H]-proline incorporation, but prevented the stimulatory effect of 10 µM carbachol on [^{3}H]-proline incorporation (Fig. 5B).

Nicotine failed to affect significantly [^{3}H]-proline incorporation. In presence of 1, 10 and 100 µM nicotine [^{3}H]-proline incorporation amounted to 103 ± 9%, 119 ± 6% and 117 ± 10% of respective controls (each n=6).

Muscarinic stimulation of collagen synthesis in HEL-299 and primary human lung fibroblasts

In HEL-299 cells basal [^{3}H]-proline incorporation (14,915 ± 557 d.p.m., n=18) was not significantly different from that observed in MRC-5 cells, whereas in primary human lung fibroblasts it was significantly lower compared to either of the cell line (10,122 ± 745 d.p.m., n=42, $P < 0.01$). Nonetheless, in both cell types muscarinic agonists stimulated [^{3}H]-proline incorporation (Fig. 6) and the IC_{50} for carbachol obtained in primary cells (165 nM) was comparable to that in MRC-5 cells. Finally, in both, HEL-299 and the primary cells the stimulatory effect of the muscarinic agonists (10 μM oxotremorine or 10 μM carbachol) were blocked by 10 nM tiotropium (Fig. 6).

DISCUSSION

In human lung fibroblasts (primary cells and different cell lines) mRNA encoding multiple muscarinic receptors was found to be expressed. However, in all cells studied so far M_2 receptor mRNA was by far the most prominent transcript. In this regard, our previous observations on primary human lung fibroblasts and MRC-5 cells [14] were confirmed in the present study in a lager series of experiments and extended to HEL-299 cells (Fig. 1), another human lung fibroblast cell line [21]. In agreement to our observations two other recent studies demonstrated in primary human lung fibroblasts muscarinic receptor mRNA expression and observed also a predominant expression of mRNA encoding the M_2 subtype [23,24].

In first functional studies it had been observed in both, primary lung fibroblasts and MRC-5 cells that muscarinic receptor activation can exert proliferative effects [14,15,17]. The present study aimed to explore further the functional significance of muscarinic receptors in

pulmonary fibroblasts and studied possible effects on collagen synthesis in MRC-5 and HEL-299 cells as well as in primary lung fibroblasts.

Collagens are proteins of particular high proline content and [^{3}H]-proline incorporation into newly synthesized proteins has often been used as a measure of collagen synthesis [e.g. 16-20]. In the present experiments digestion with collagenase confirmed that [^{3}H]-proline incorporation largely reflects collagen synthesis (Fig. 3A) and that drug effects on total [^{3}H]-proline incorporation paralleled those on the collagenase-sensitive [^{3}H]-proline fraction (Fig. 3B). Moreover, stimuli known to inhibit (butaprost and forskolin) or to stimulate (TGF-ß$_1$) collagen synthesis [20,25] showed corresponding effects also in the present study.

In MRC-5 cells carbachol as well as oxotremorine, two well known muscarinic receptor agonists, caused a clear increase in collagen synthesis. In contrast to oxotremorine, which is a pure muscarinic agonist, carbachol has also some nicotine receptor agonistic activity. However, nicotine up to 100 µM did not significantly affect [^{3}H]-proline incorporation which argues against a role of nicotine receptors in the effect of carbachol. Finally, interaction experiments, in which the effect of carbachol was antagonized by the long-acting muscarinic antagonist tiotropium (Fig. 4) proved the mediation via specific muscarinic receptors.

In previous experiments it was observed that the muscarinic receptors mediating proliferative effects in lung fibroblasts had pharmacological characteristics of M_2 receptors and coupled to pertussis toxin sensitive G proteins [14]. Likewise, the muscarinic stimulation of collagen synthesis was also prevented by pertussis toxin (Fig. 5) indicating the involvement of $G_{i/o}$ proteins also in this effect. In the present experiments, a detailed pharmacological characterisation of the muscarinic receptors mediating stimulatory effects on collagen synthesis was not performed, but of the five muscarinic receptor subtypes only M_2 and M_4 receptors couple to pertussis toxin sensitive proteins [26]. Moreover, MRC-5 cells express substantial amounts of transcript for M_2 receptors, but only traces of mRNA encoding M_4 receptors (Fig. 1). Thus, M_2 receptors are likely to mediate also the muscarinic stimulation of

collagen synthesis. Is this conclusion compatible with the high potency of tiotropium to antagonize the stimulatory effect of carbachol on collagen synthesis? Affinity constants of tiotropium are known for M_1, M_2, and M_3 receptors and are similar for all three receptor subtypes (0.1 – 0.3 nM) [27,28]. However, the dissociation of the respective receptor complexes shows different kinetics, with the longest half-life for the tiotropium-M_3 receptor complex [27,28]. This kinetic subtype selectivity of tiotropium may not be of significance in the present experiments, because they were performed under equilibrium conditions. On the other hand. whether the kinetical subtype selectivity of tiotropium is of clinical significance is still a matter of debate [29]. In any case, it is very likely that under clinical conditions tiotropium causes a significant blockade of M_2 receptors in the lung, in addition to the blockade of M_3 and M_1 receptors, because the half-life of the tiotropium–M_2 receptor complex, although shorter than that of those of M_3 and M_1 receptors, is with 3.6 h still quite long compared with ipatropium for which the dissociation half-lifes are in the range of 2-6 minutes for all three muscarinic receptors [27,28].

Furthermore, in both MRC-5 cells and the primary human lung fibroblasts the muscarinic proliferative effects were mediated via activation of the ERK-MAP kinase pathway [15]. The present observations showing that PD 98059 prevented the carbachol-induced stimulation of [^{3}H]-proline incorporation indicate that this pathway is also essential for the muscarinic stimulation of collagen synthesis.

As already mentioned above, also in HEL-299 and primary human lung fibroblasts mRNA encoding M_2 receptors is the predominant muscarinic receptor transcript. In the present experiments muscarinic agonists stimulated collagen synthesis also in these cells. Thus, similar to observations in previous studies in which proliferative effects were studied [14,15] muscarinic receptor activation elicits the same response in primary human lung fibroblasts and human lung fibroblast cell lines.

In conclusion, the present results demonstrate muscarinic stimulation of collagen synthesis in cultured human lung fibroblasts. Together with previous observations showing muscarinic receptor-mediated proliferative effects [14,15,23], the present findings support the notion that cholinergic mechanisms could play a role in pro-fibrotic airway remodelling processes which occur in chronic inflammatory and obstructive airway diseases. Inhibition of these processes by prolonged blockade of airway muscarinic receptors may contribute to the long-term beneficial effects of anticholinergics, as observed for example for the long-acting muscarinic antagonist tiotropium in COPD.

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Fig. 1 *Upper part:* Samples of RT-PCRs of human muscarinic receptors (M_1-M_5) on RNA isolated from different human lung fibroblasts (primary cells (phLFb) and the cell lines MRC-5 and HEL-299). Cells were grown in 55 mm culture dishes to confluency, total RNA was isolated, treated with DNase and used for RT-PCR with primers specific for the human muscarinic receptors or ß-actin. To control effectiveness of primer pairs, PCRs were also performed on genomic DNA (gDNA) isolated from MRC-5 cells. One PCR lacking template DNA was regularly performed to exclude any contamination (Neg.). PCR products were separated on a 1.2% agarose gel. *Lower part:* Densitometrical evaluation of a series of experiments. Density values (arbitrary units) were first normalised over ß-actin to correct for quality of the cDNA preparation and second over the respective amplification product obtained on gDNA to correct for effectiveness of the respective primer pair *A:* Expression of mRNA encoding M_2 receptors in absolute terms. *B-D:* Expression of mRNA for M_1 - M_5 receptors, expressed as % of the M_2 receptor value of each individual cell preparation. Given are individual observations and mean values (horizontal bar) (for cell lines each dot gives an observation from a separate cell preparations, for phLFb each dot gives an observation from cells obtained from an individual patient).

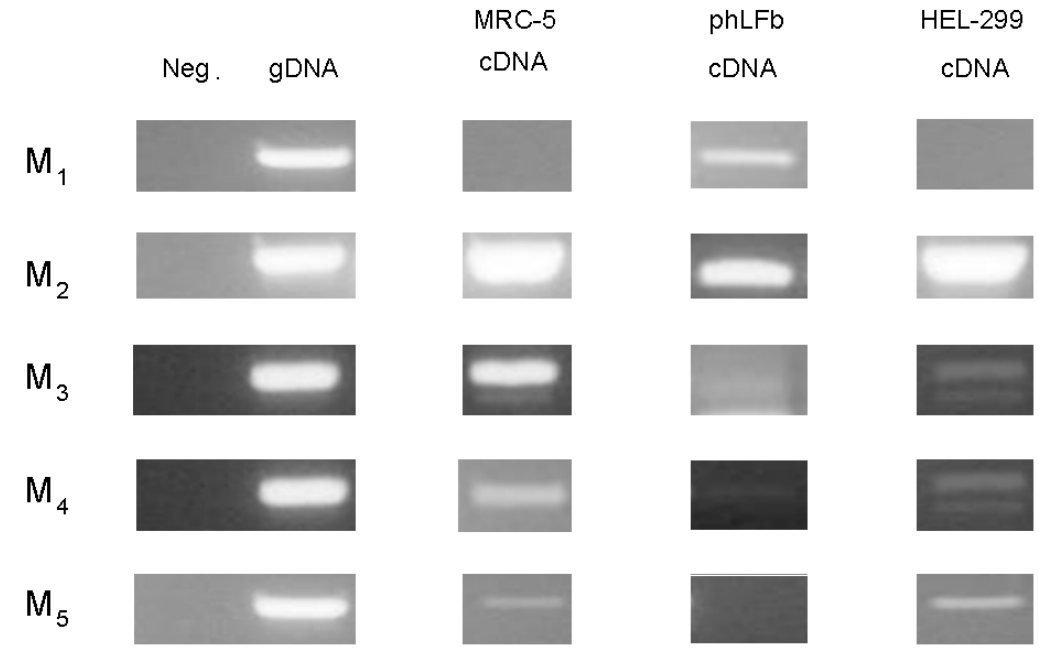
Fig. 2 Effects of carbachol (Carb), oxotremorine (Oxo) (A) and butaprost (Buta), forskolin (Fors) and TGF-$ß_1$ (B) on collagen synthesis in human lung fibroblasts (MRC-5 cells) as determined by [^{3}H]-proline incorporation. After dissemination, cells were cultured for 24 h in presence of 10% FCS followed by 24 h under FCS-deprived conditions. Thereafter, [^{3}H]-proline (37 kBq) together with test drugs was added for additional 24 h under FCS-free conditions. Incorporation of [^{3}H]-proline into cellular proteins is expressed as % of the mean value of the respective controls of each individual cell preparation. Given are individual observations and mean values (horizontal bar).

Fig. 3 Comparison of the effects of butaprost (Buta) and carbachol (Carb) on total and collagenase-sensitive [^{3}H]-proline incorporation into cellular proteins synthesized by human lung fibroblasts (MRC-5 cells) cultured as described in Fig. 2. Cellular proteins were extracted and aliquots of the protein extracts were incubated for 1 h at 37°C in collagenase II (2 mg/ml) containing buffer and the releasable [^{3}H]-proline was determined. *A,* [^{3}H]-proline released by collagenase treatment, expressed as % of total protein [^{3}H] in each individual experiment, pooled data of the experiments shown in part B. *B,* Effects of Buta and Carb on [^{3}H]-proline incorporation, measured as total [^{3}H]-incorporation and collagenase sensitive [^{3}H]-incorporation, expressed as % of the mean value of the respective controls of each individual cell preparation. Given are individual observations and mean values (horizontal bar).

Fig. 4 Concentration-dependent inhibitory effects of tiotropium on carbachol-induced stimulation of collagen synthesis by human lung fibroblasts (MRC-5 cells), as determined by [^{3}H]-proline incorporation. Cells were cultured as described in Fig. 2. Tiotropium in the concentrations given was present 30 min prior to the addition of [^{3}H]-proline and carbachol. [^{3}H]-proline incorporation is expressed as % of the mean value of the respective carbachol-induced increase in the respective cell preparation. Given are individual observations and mean values (horizontal bar). The mean carbachol-induced increase in this series of experiments amounted to 54±4%.

Fig. 5 Effects of carbachol (Carb) and/or pertussis toxin (PTX) (A) and/or PD 98059 (B) on collagen synthesis by human lung fibroblasts (MRC-5 cells) as determined by [^{3}H]-proline incorporation. Cell were cultured as described in Fig. 2. PTX (0.1 μg/ml) was present 3 h and PD 98059 30 min prior to the addition of [^{3}H]-proline and Carb (10 μM). Incorporation of [^{3}H]-proline into cellular proteins is expressed as % of the mean value of the respective controls of each individual cell preparation. Given are individual observations and mean values (horizontal bar).

Fig. 6 Effects of muscarinic agonists in absence and presence of tiotropium on collagen synthesis by HEL-299 (A) and primary human lung fibroblasts (B) as determined by [^{3}H]-proline incorporation. Primary cells were obtained from lung tissue of 4 patients and cells from each patient were used in different passages (3-11) for experiments. Cells were cultured as described in Fig. 2 for MRC-5 cells. Tiotropium (Tio, 10 nM) was present 30 min prior to the addition of [^{3}H]-proline and the agonists (oxotremorine (Oxo (10 μM) or carbachol (at concentrations given)). Incorporation of [^{3}H]-proline into cellular proteins is expressed as % of the mean value of the respective controls of each individual cell preparation. Given are individual observations and mean values (horizontal bar).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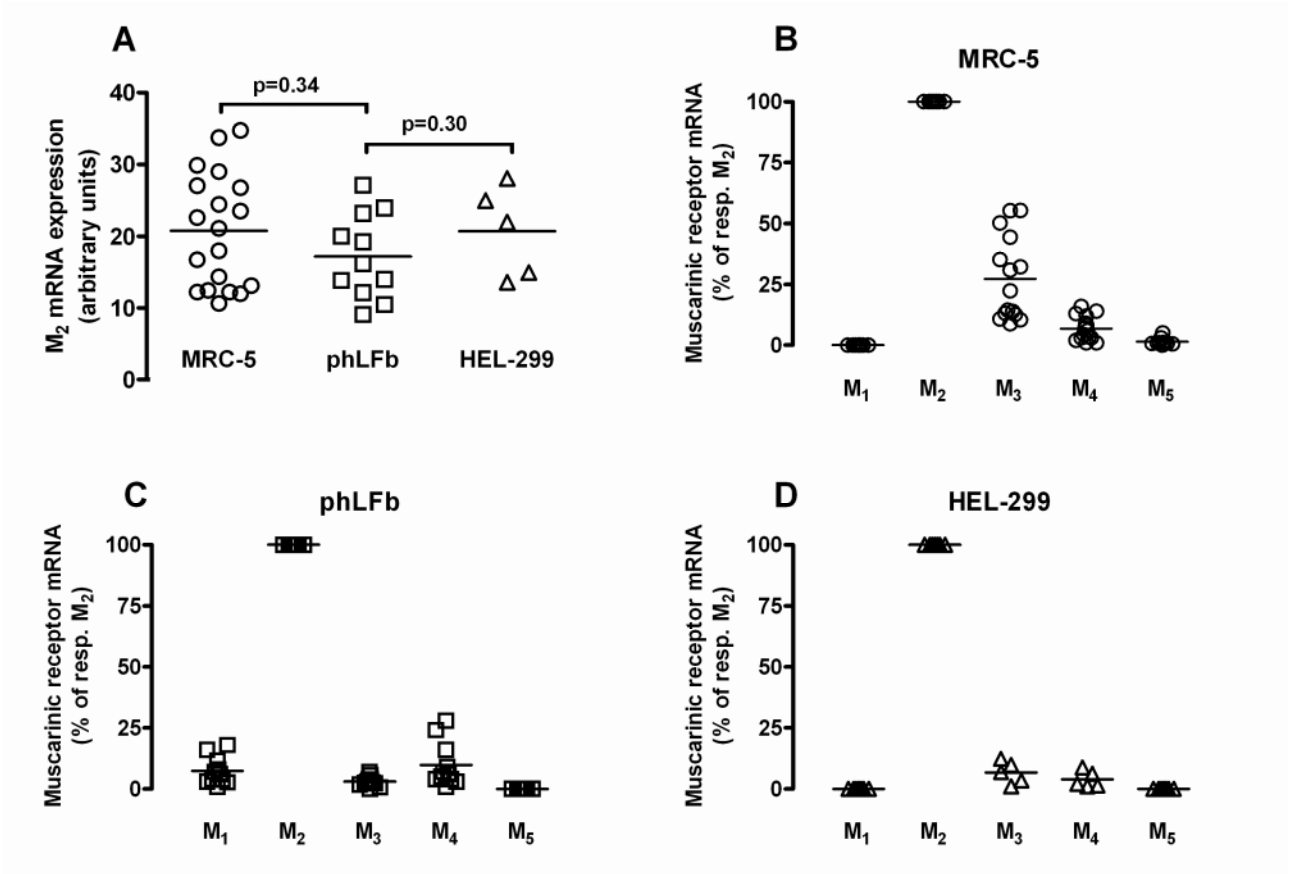
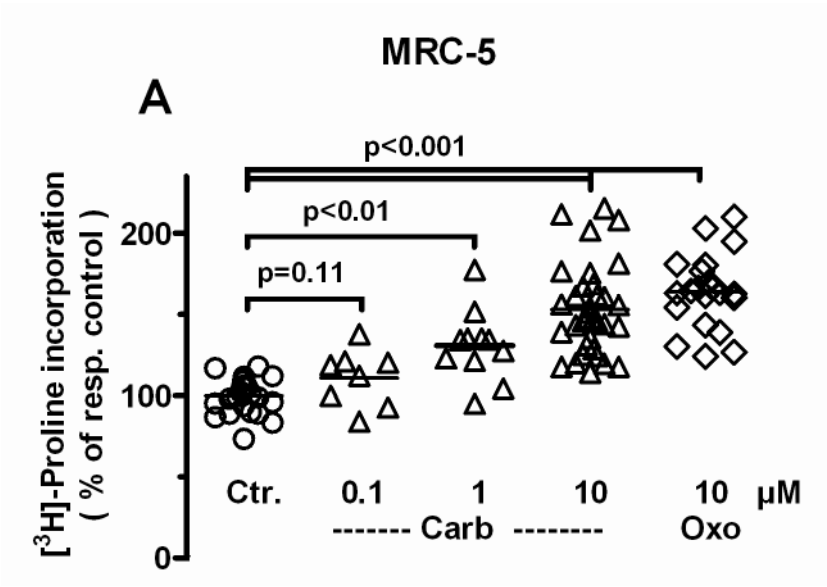

A
p=0.34
p=0.30
M2 mRNA expression (arbitrary units)
MRC-5
phLFb
HEL-299
B
MRC-5
Muscarinic receptor mRNA (% of resp. M2)
C
phLFb
D
HEL-299
M1
M2
M3
M4
M5

Fig. 1


MRC-5
A
p<0.001
p<0.01
p=0.11
[3H]-Proline incorporation
(% of resp. control)
200
100
0
Ctr.
0.1
1
10
10 µM
------- Carb --------
Oxo

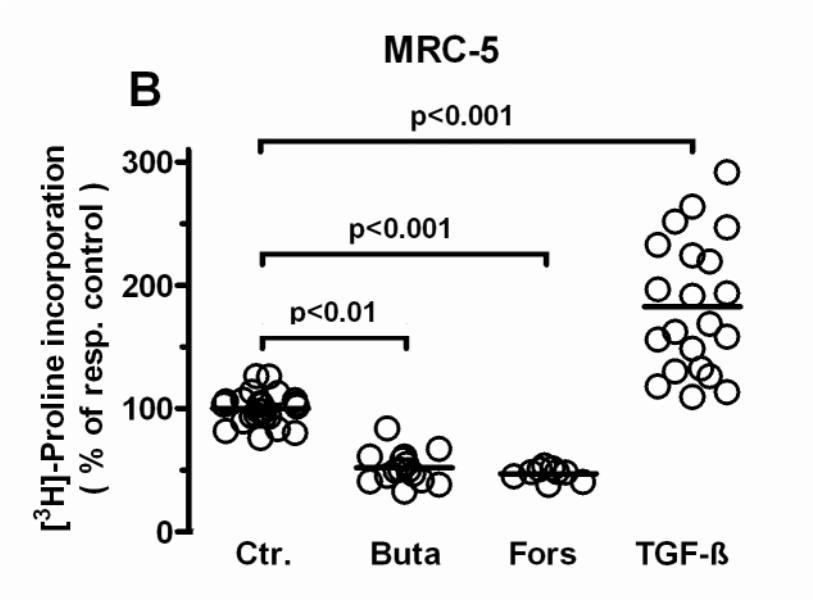

MRC-5
B
p<0.001
p<0.001
p<0.01
[3H]-Proline incorporation
(% of resp. control)
300
200
100
0
Ctr.
Buta
Fors
TGF-ß

Fig.2

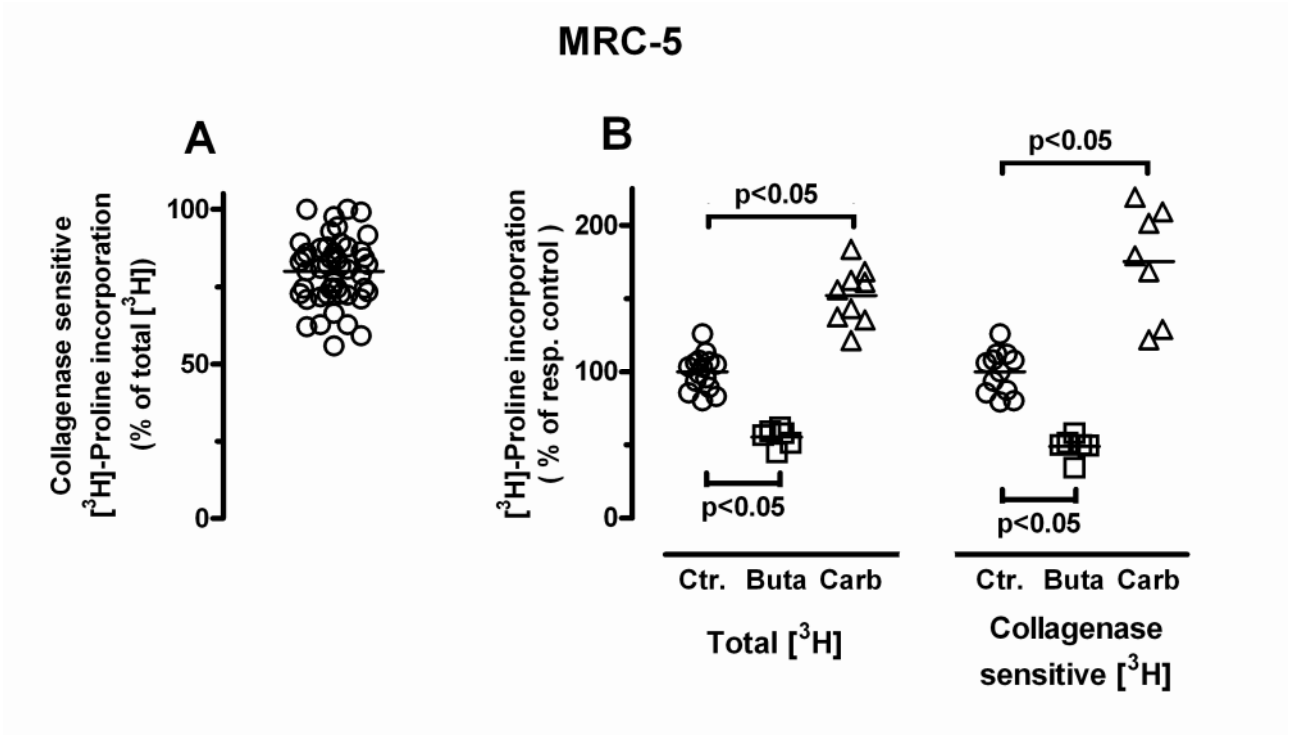

MRC-5
A
B
Collagenase sensitive [3H]-Proline incorporation (% of total [3H])
100
50
0
[3H]-Proline incorporation (% of resp. control)
200
100
0
p<0.05
p<0.05
p<0.05
p<0.05
Ctr. Buta Carb
Total [3H]
Ctr. Buta Carb
Collagenase sensitive [3H]

Fig. 3

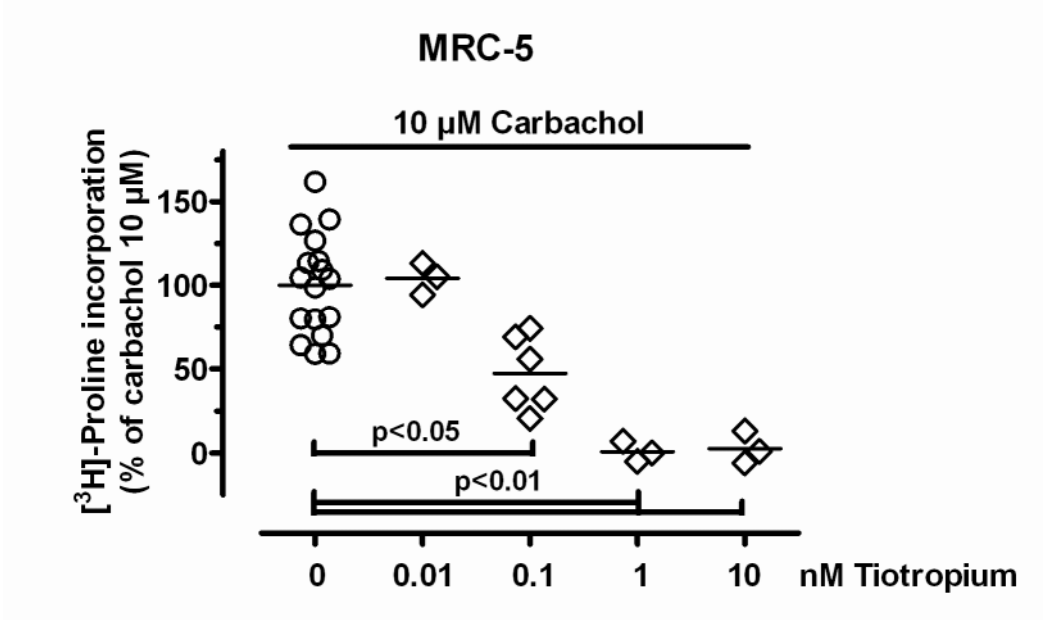

MRC-5
10 µM Carbachol
[3H]-Proline incorporation (% of carbachol 10 µM)
150
100
50
0
p<0.05
p<0.01
0
0.01
0.1
1
10
nM Tiotropium

Fig. 4

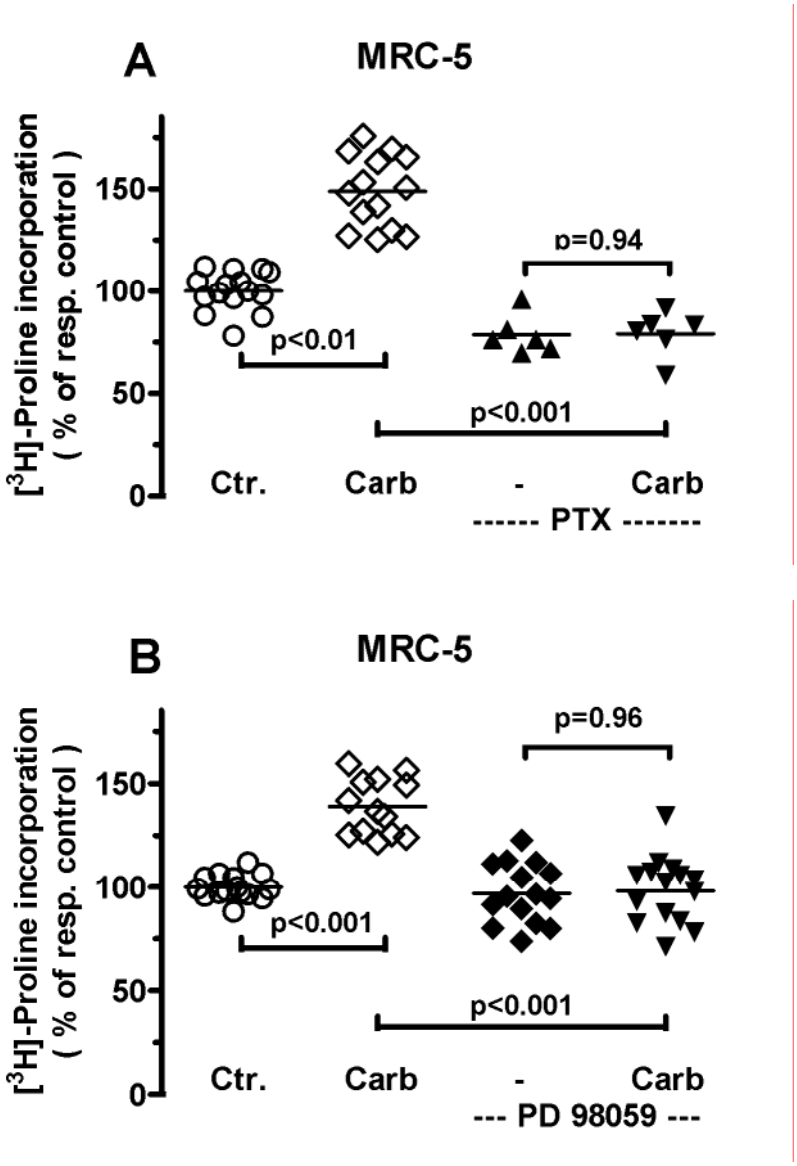

A
MRC-5
[3H]-Proline incorporation
(% of resp. control)
150
100
50
0
p=0.94
p<0.01
p<0.001
Ctr.
Carb
-
Carb
------ PTX -------
B
MRC-5
[3H]-Proline incorporation
(% of resp. control)
150
100
50
0
p=0.96
p<0.001
p<0.001
Ctr.
Carb
-
Carb
--- PD 98059 ---

Fig. 5

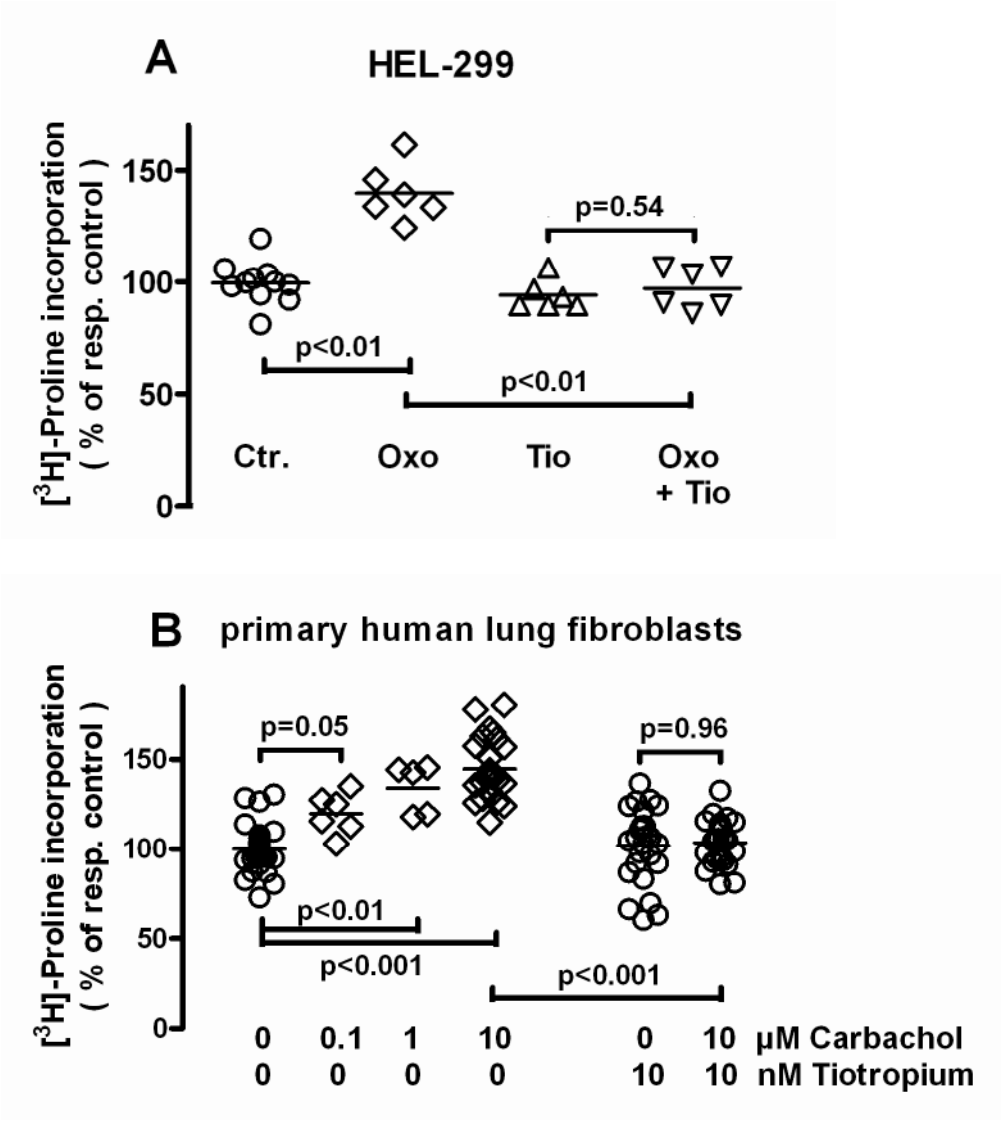

A
HEL-299
[3H]-Proline incorporation
(% of resp. control)
150
100
50
0
p=0.54
p<0.01
p<0.01
Ctr.
Oxo
Tio
Oxo
+ Tio
B
primary human lung fibroblasts
[3H]-Proline incorporation
(% of resp. control)
150
100
50
0
p=0.05
p=0.96
p<0.01
p<0.001
p<0.001
0 0.1 1 10 0 10 µM Carbachol
0 0 0 0 10 10 nM Tiotropium

Fig. 6