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Original article

The muscarinic antagonist gallamine induces proliferation of airway smooth muscle cells regardless of the cell phenotype

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ABSTRACT

Background: Muscarinic receptor antagonists are a usual treatment for chronic airway diseases, with increased bronchoconstriction, like asthma and chronic obstructive pulmonary disease. These diseases are usually accompanied by airway remodeling, involving airway smooth muscle cell (ASMC) proliferation. The purpose of this study was to examine the effect of the muscarinic receptor modulator gallamine on rabbit tracheal ASMC proliferation.
Methods: ASMCs were incubated with gallamine (1 nM–10 mM), atropine (1 fM–10 mM), and/or acetylcholine (1 nM–1 mM), in the presence or absence of FBS (1% or 10%). Cell proliferation was estimated by incorporation of radioactive thymidine, the Cell Titer AQueous One Solution method and cell number counting after Trypan blue exclusion. The mechanisms mediating cell proliferation were studied using the PI3K and MAPK inhibitors LY294002 (20 μM) and PD98059 (100 μM), respectively. Cell phenotype was studied by indirect immunofluorescence for α-actin, Myosin Heavy Chain and desmin.
Results: ASMC incubation with the muscarinic receptor allosteric modulator gallamine or the muscarinic receptor antagonist atropine increased methyl-[^3H]thymidine incorporation and cell number in a dose-dependent manner. ASMC proliferation was mediated *via* PI3K and MAPK activation and was transient. Gallamine antagonized the mitogenic effect of 1% FBS. Furthermore, gallamine had a similar effect on contractile ASMCs, without synergizing with or affecting acetylcholine induced proliferation, or altering the percentage of ASMCs expressing contractile phenotype marker proteins.
Conclusions: Gallamine, in the absence of any agonist, has a transient mitogenic effect on ASMCs, regardless of the cell phenotype, mediated by the PI3K and the MAPK signaling pathways.

Introduction

Airway wall remodeling is a common symptom in many chronic respiratory diseases, like asthma and Chronic Obstructive Pulmonary Disease (COPD) [1]. Airway wall remodeling is a complex phenomenon, which includes a variety of structural changes, such as thickening of the basal membrane, increase in the number and the size of vessels, as well as hyperplasia and hypertrophy of airway smooth muscle [2–4]. As this increase of the airway smooth muscle cell (ASMC) mass leads to the reduction of the airway diameter and restriction of the amount of air that reaches the lungs, the physiological, inflammatory or pharmaceutical factors that affect it, are subject to extensive and interesting research.

ASMC proliferation is mainly mediated by the activation of metabolic signaling pathways. The 3-phoshatidiloinositol kinase (PI3K) and the mitogen activated protein kinase (MAPK) pathways, have been shown to respond to various proliferative stimuli and play a central role through the induction of genes that lead to DNA synthesis and mitotic division [4,5]. It is interesting that the activation of these pathways, and hence proliferation, depends on the nature, duration and intensity of different mitogenic stimuli, as well as the spatiotemporal expression of responding receptors such as growth factor receptors and muscarinic receptors to name but a few [5,6].

ASMCs express on their membrane muscarinic receptors. Muscarinic receptors are G-coupled receptors and are divided to 5 different subtypes: M_1–M_5. The expression of each particular receptor subtype depends on the species, the tissue type [6] as well as the cell phenotype [7]. M_3 receptors, located on ASMCs, are responsible for the responsiveness to cholinergic stimulus [8]. The expression of M_2 receptors on ASMC membranes has also been confirmed [9–11] but the physiological significance of their presence on airway smooth muscle is still unknown. The activation

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of G proteins through M_2 or M_3 receptors could lead to ASMC contraction, *via* intracellular stores Ca^{2+} release [12]. Moreover, it has been found that ASMCs express the ACh synthesizing enzyme choline acetyltransferase (ChAT), and presumably synthesize ACh [13,14]. Furthermore, it has been observed that muscarinic agonists induce proliferation of ASMCs *via* PI3K and MAPK signaling pathways dependent mechanisms [7,12] and that, the physiological signaling of muscarinic receptors and cholinergic transmission is altered at respiratory diseases [15,16].

Muscarinic receptors antagonists, referred to as anticholinergics, are common drugs that cause bronchodilation and are used widely, alone or in combination with long acting β_2 agonists, for the treatment of mainly of COPD and, to a lesser extent, of asthma. Currently used anticholinergic drugs, such as tiotropium, are antagonists of the M_2 and/or M_3 muscarinic receptors [17,18]. On the other hand, limited data suggest that muscarinic receptor allosteric modulators such as alcuronium, gallamine and strychnine may not only inhibit agonist mediated effects but also stimulate cell signaling, in the absence of an agonist through allosteric binding to the receptor [19,20].

As M_2/M_3 antagonists are commonly used drugs, we sought to examine their effect on ASM cell proliferation and phenotype. For this purpose we used gallamine, an allosteric modulator with a selectivity on $M_2>M_1$, $M_4>M_3$, M_5 receptors, studied its' effects on the proliferation of rabbit tracheal ASMC, and compared them to that of the nonspecific muscarinic receptor antagonist atropine. The signaling pathways involved in the effect of gallamine were also studied in an attempt to elucidate the mechanisms involved.

Materials and methods

Ethical approval

Adult New Zealand rabbits used for the study were maintained in a proper environment and treated in compliance with ethical and institutional guidelines. The protocol used has been approved by the Bioethics Committee of the Department of Medicine, School of Health Sciences of the University the study was performed in. An overdose of intravenously administrated Pentothal (Abbott, Abbott Park, IL, USA) was used for euthanasia.

Airway smooth muscle cells (ASMC) isolation and culture

We used a previously described protocol in order to isolate ASMC from adult rabbit trachea [21]. Isolated cells were washed and centrifuged (1000 rpm for 10 min) twice in Dulbecco's modified Eagle's medium/Ham/F12 (DMEM/F12) with 10% fetal bovine serum (FBS), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, placed in culture flasks and cultured at 37 °C with 5% CO_2. Cells used for experiments were at passages 2–4.

ASMC treatment

Cells were rinsed twice with PBS and incubated in FBS-free DMEM/F12, 100 U/ml penicillin, 100 μg/ml streptomycin for 24 h, 7 or 15 days. ASMCs were exposed to the muscarinic modulator gallamine (1 nM–10 mM), the nonspecific antagonist atropine (1 fM–10 mM), 1% or 10% FBS or the muscarinic agonists acetylcholine (1 nM–1 mM), as indicated. Cells were treated with LY294002 (20 μM) for 15 min or with PD98059 (100 μM) for 1 h, prior to the incubation with gallamine.

Estimation of ASMC proliferation

Cell Titer 96® AQueous One Solution Assay (Promega) method

The reduction of the MTT chromophore by the mitochondria of the living cells was measured at 490 nm with a reference at 630 nm, using an ELISA plate reader.

Methyl-[^3H] thymidine incorporation.

Cells were incubated in the presence of methyl-[^3H] thymidine for 18 h, and the incorporation of radioactive material in DNA, synthesized during DNA replication in proliferating cells [21], was

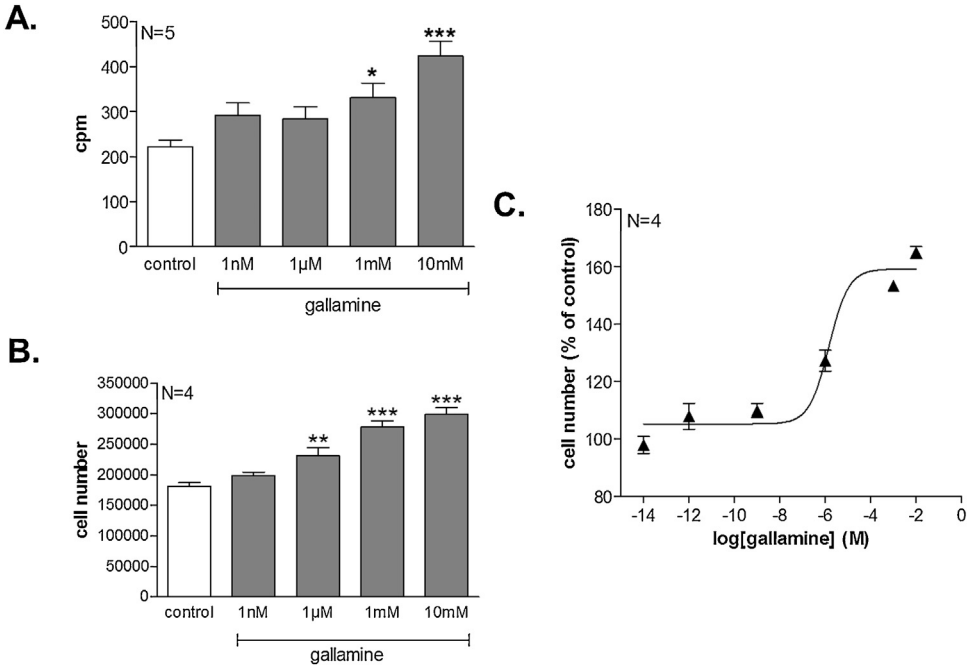

Fig. 1. The muscarinic receptor modulator gallamine induces proliferation of ASMC. ASMCs were serum starved for 24 h prior to incubation in the presence of gallamine (1 nM–10 mM) for 24 h. (A) Measurement of methyl-[^3H]thymidine incorporation. (B) Viable ASMC count after Trypan blue staining. (C) Dose -response analysis of the effect of gallamine on ASMC number. Data are presented as mean ± SEM of 4–5 independent experiments, as indicated (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to control cells incubated in the absence of gallamine).

measured in a Wallac scintillation counter and expressed as counts/min.

Cell counting with Trypan blue exclusion method.

ASMC were detached from the culture dish and suspended in PBS. Cell number was estimated using a Neubauer heamocytometer. Dead cells were excluded by Trypan blue staining.

Indirect immunofluoresence

ASMCs were plated onto coverslips and treated as indicated above for 15 days. Contractile protein expression was evaluated with the use of anti-smooth muscle α-actin mouse monoclonal antibody (1:1000, Sigma), anti-myosin (smooth) clone HSM-V mouse monoclonal antibody (1:1000, Sigma) and anti-desmin rabbit polyclonal antibody (1:1000, Sigma). Prior to incubation with the antibodies PBS-3% formaldehyde, PBS-1% Triton X-100 and PBS-0.1% Tween 20-3% BSA were used for fixation, permeabilation and blocking, respectively. Following incubation with anti-mouse IgG or anti-rabbit IgG antibodies conjugated with CY3, the coverslips were mounted on Vectrashield solution containing DAPI for DNA staining. The background was removed from all the photos taken from the indirect immunofluorescence experiments and cells with a signal above background were characterized as positive. The results represent the mean measurements of 2 independent researchers.

Statistics

In experiments where cell proliferation was estimated, each point was performed 3 times and the values represent the mean of 4–7 independent experiments. In the indirect immunofluorescence the values presented are the mean of 3 independent experiments. All data are expressed as means ± standard error of the mean (SEM) and the number of experiments performed is stated by N values. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Differences between means were analyzed using one-way ANOVA followed by Bonferonni's *post*-test. Results were considered significantly different when $p < 0.05$. Half maximal effective concentration (EC_{50}) and maximal effect (B_{max}) values were estimated with nonlinear regression analysis.

Results

The proliferation of ASMCs, serum-starved for 24 h and incubated for another 24 h in the presence of the muscarinic receptor allosteric modulator gallamine or the muscarinic receptor antagonist atropine, was investigated by measuring DNA synthesis and cell number. Gallamine (1 nM – 10 mM) had a dose-dependent proliferative effect on ASMCs (Fig. 1), as shown by the increase in both methyl-[^3H]thymidine incorporation (Fig. 1A) and cell number (Fig. 1B). Indicatively, incubation in the presence of 10 mM gallamine increased methyl-[^3H]thymidine incorporation from 221.9 ± 15.62 to 424 ± 32.61 cpm ($p < 0.001$, Fig. 1A) and cell number approximately 1.6 fold from 181,250 ± 5846 to 298,958 ± 10,999 cells ($p < 0.001$, Fig. 1B). The respective gallamine mitogenic effect EC_{50} value was 1.45 μM and the B_{max} value 159 ± 2.7% of control cells (Fig. 1C).

Interestingly, the nonselective muscarinic antagonist atropine (1 nM–10 mM) increased methyl-[^3H]thymidine incorporation only in the low concentration of 1 nM from 224.3 ± 19.98 to 423.4 ± 68.71 cpm ($p < 0.01$, Fig. 2A), while incubation of ASMCs with 10 mM atropine significantly decreased methyl-[^3H]thymidine incorporation ($p < 0.05$, Fig. 2A). Further analysis using lower atropine concentrations (1 fM-1 μM), revealed that incubation of ASMCs with atropine (1 pM – 1 nM) increased proliferation in a dose dependent manner, as shown by assessing ASMC number; e.g. 1 pM atropine increased cell number from 189,063 ± 10,325 to 253,125 ± 7439 ($p < 0.05$, Fig. 2B). As expected, neither gallamine nor atropine had a proliferative effect, when used in the presence of 10% FBS (Fig. 3A).

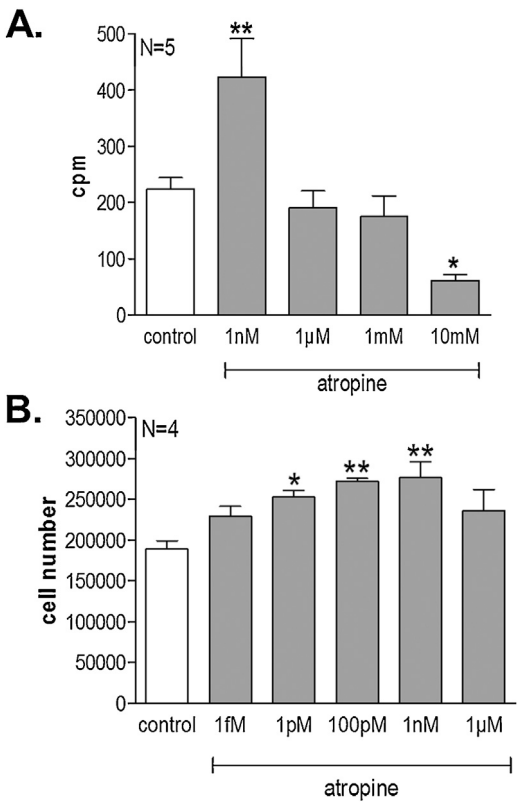

Fig. 2. The muscarinic receptor antagonist atropine induces proliferation of ASMCs. ASMCs were serum starved for 24 h prior to incubation in the presence of atropine for another 24 h. (A) Measurement of methyl-[^3H]thymidine incorporation in ASMCs treated with atropine (1 nM–10 mM). (B) Number of viable ASMCs after treatment with atropine (1 fM–1 μM). Data are presented as mean ± SEM of 4–5 independent experiments, as indicated. (*$p <$ 0.05 and **$p <$ 0.01 compared to control cells incubated in the absence of atropine).

As the proliferation induced by 10% FBS is generally considered to be maximal, we further studied the possible additive effect of gallamine (10 mM) on the proliferation of ASMCs induced by lower concentrations of FBS. For this purpose, we measured methyl-[^3H]thymidine incorporation on ASMCs serum-starved for 24 h and subsequently incubated in the presence or absence of 1% FBS and/or gallamine for 2 or 6 days (Fig. 3B). Gallamine as well as 1% FBS increased methyl-[^3H]thymidine incorporation. On the contrary, the addition of gallamine in culture medium reduced significantly ($p < 0.01$) the mitogenic effect of 1% FBS (Fig. 3B). Namely, after 48 h treatment, methyl-[^3H]thymidine was 2702 ± 183.2 cpm in the presence of 1% FBS, 1291 ± 31.75 cpm in the presence of 10 mM gallamine and 1784 ± 316.5 cpm when both 1% FBS and 10 mM gallamine were supplied together. Interestingly, addition of gallamine in the cell culture medium caused an increase in methyl-[^3H]thymidine incorporation that reached a maximum after 2 days and did not increase further after 6 days (Fig. 3B). In contrast, methyl-[^3H]thymidine incorporation continued to increase in ASCMs incubated in medium containing 1% FBS (Fig. 3B). This lack of a time dependent increase of cell proliferation probably indicates that the effect of gallamine is transient or is exhausted within the first 24 h of incubation.

To elucidate the mechanism mediating the mitogenic effect of gallamine, we studied the involvement of the PI3K and MAPK

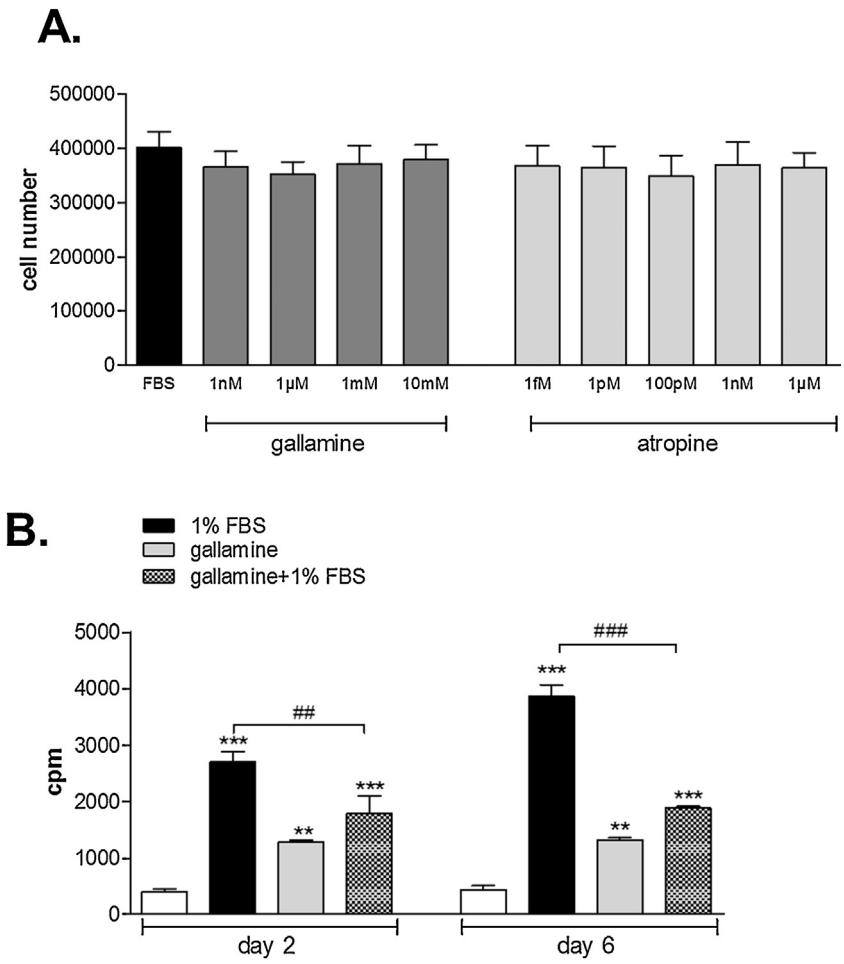

Fig. 3. Effect of gallamine and atropine on ASMC proliferation in the presence of FBS. (A) Gallamine and atropine do not affect ASMC proliferation in the presence of 10% FBS. Number of viable ASMCs after treatment with gallamine (1 nM–10 mM) or atropine (1 fM–1 μM) in the presence of 10% FBS. Data are presented as mean ± SEM of 4 independent experiments. (B) Gallamine has a transient effect on ASMC proliferation and decreases the mitogenic effect of 1% FBS. Methyl-[^3H]thymidine incorporation in ASMCs incubated for 24 h in serum free medium, prior to the addition of 1% FBS for 2 or 6 days in the presence or absence of gallamine (10 mM). Data are presented as mean ± SEM of 4 independent experiments (**$p < 0.01$ and ***$p < 0.001$ compared to relative control, ## $p < 0.01$ and ### $p < 0.001$ for comparison between cells incubated with 1% FBS or the combination of 1% FBS and 10 mM gallamine).

signaling pathways. Incubation of ASMCs with the PI3K pathway inhibitor LY294002 (20 μM) significantly decreased methyl-[^3H] thymidine incorporation, from 404.9 ± 87.52 cpm to 201.1 ± 39.96 cpm ($p < 0.05$, Fig. 4). A similar reduction was observed in the presence of the MAPK pathway inhibitor PD98059 (100 μM) ($p < 0.05$, Fig. 4) indicating that the gallamine-induced ASMC proliferation is mediated by the PI3K and MAPK signaling pathways. The inhibitors alone had no effect on ASMC proliferation in the absence of FBS while they significantly inhibited cell proliferation in the presence of 10% FBS as was shown previously [22].

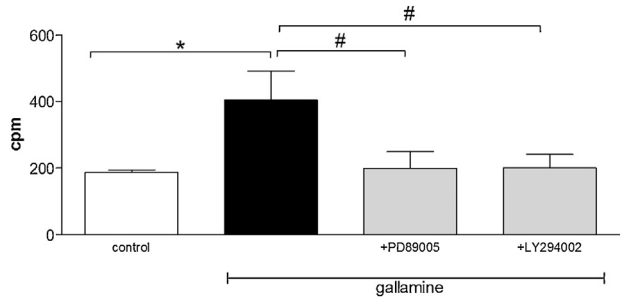

Fig. 4. The gallamine induced ASMC proliferation is blocked by PI3K and MAPK signaling pathway inhibitors. Methyl-[^3H]thymidine incorporation in ASMCs incubated in serum-free medium for 24 h and then incubated for 24 h with gallamine (10 mM), in the presence or absence of the PI3K pathway inhibitor LY294002 (20 μM) or the MAPK pathway inhibitor PD98059 (100 μM), as indicated. Data are presented as mean ± SEM of 6 independent experiments (*$p < 0.05$). # $p < 0.05$, compared to gallamine alone.

Prolonged serum starvation induces the differentiation of ASMCs towards a contractile phenotype [7]. To study the effect of gallamine on contractile ASMC after incubation in FBS free medium for 7 days, cells were treated with acetylcholine alone (1 nM–1 mM) or acetylcholine in the presence of gallamine (10 mM) for 24 h and cell proliferation was assessed by counting cell number after Trypan blue staining. As expected, acetylcholine alone had a dose-dependent mitogenic effect on ASMCs (Fig. 5A) with an EC_{50} value of 0.63 μM and a B_{max} value of 188 ± 3.7% of control cells. Gallamine (10 mM) did not alter the mitogenic effect of acetylcholine (Fig. 5A). In the presence of gallamine, the acetylcholine EC_{50} value was 0.76 μM and the B_{max} value 178 ± 5% of control cells. Interestingly, acetylcholine (1 nM-1 mM) decreased the proliferative effect of 10 mM gallamine in a dose dependent manner and this effect was more apparent at the low concentrations of acetylcholine and decreased at higher concentrations (Fig. 5B). To study the effect of gallamine in contractile cells further, ASMC were incubated in FBS free medium for 7 days, and then treated with gallamine (10 mM), the muscarinic receptor

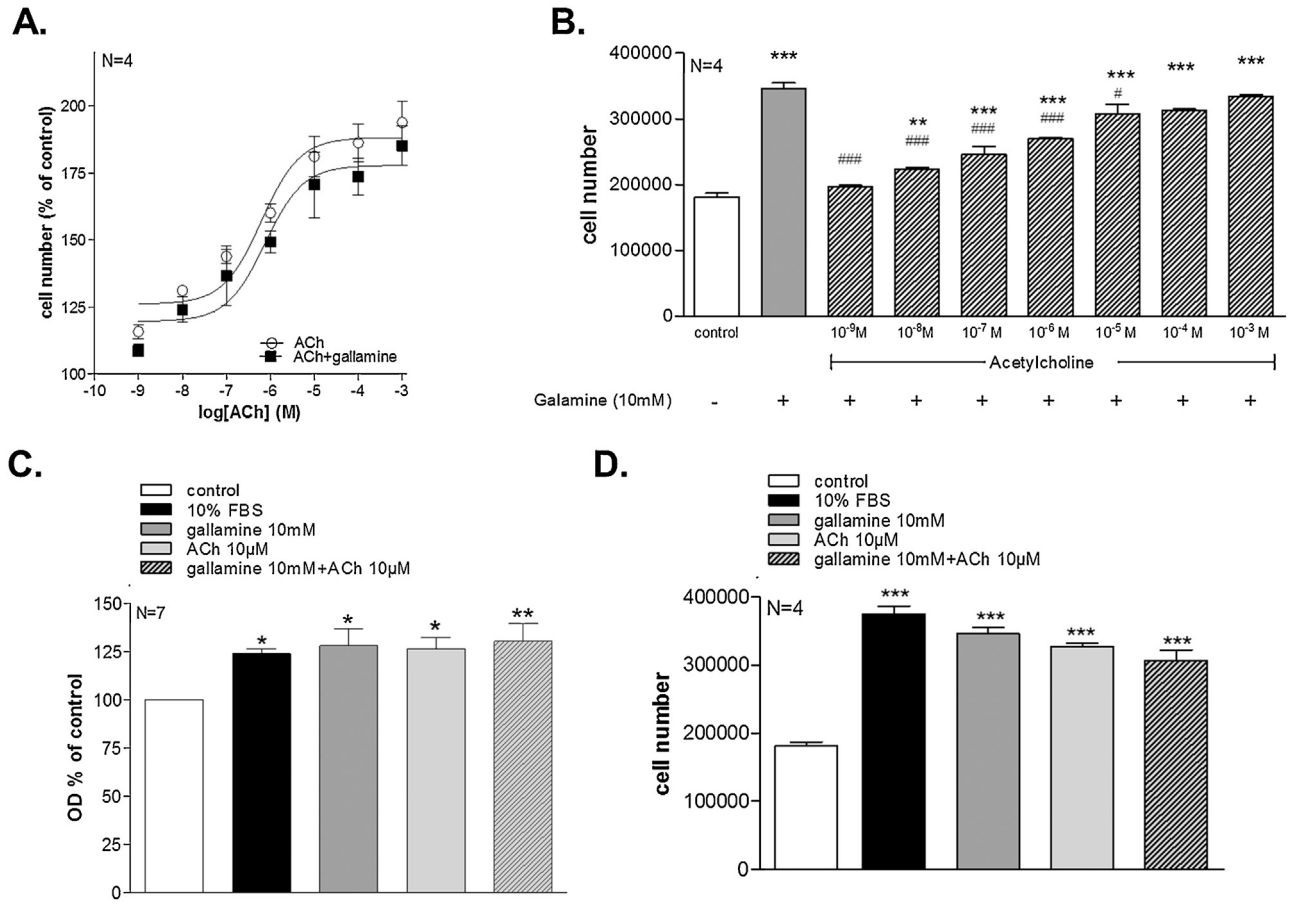

Fig. 5. Gallamine induces the proliferation of contractile ASMCs. ASMCs were incubated for 7 days in serum free medium prior to the addition of gallamine (10 mM), acetylcholine (ACh 1 nM – 10 mM) or their combination for 24 h. (A) Analysis of the effect of gallamine (10 mM) on the proliferation of contractile ASMCs incubated with achetylcholine (1 nM –1 mM). (B) Analysis of the effect of achetylcholine (1 nM –1 mM) on the proliferation of contractile ASMCs incubated with gallamine (10 mM). (C) ASMC proliferation was estimated using the Cell Titer 96® AQueous One Solution Assay. (D) ASMC number was estimated after viable cell count after Trypan blue staining. Data are presented as mean ± SEM of 4–7 independent experiments, as indicated ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control cells and $\#p < 0.05$, $\#\#\#p < 0.001$ for comparisons with cells incubated with 10 mM gallamine alone).

agonist acetylcholine (10 μM), or 10% FBS, for 24 h. Although, incubation with gallamine (10 mM) or acetylcholine (10 μM) alone had a similar mitogenic effect on serum starved ASMCs with that caused by incubation with 10% FBS, as assessed using the Cell Titer 96® AQueous One Solution Assay (Fig. 5C) or cell counting after Trypan blue staining (Fig. 5D), no additive effect on ASMC proliferation was observed when ASMCs were incubated with gallamine in the presence of acetylcholine (10 μM) (Fig. 5). Namely, the OD value for ASMCs incubated for 24 h with 10% FBS was 124.1 ± 2.5% and 133.2 ± 14.4% of control (N = 7), in the presence or absence of gallamine (10 mM) respectively.

Differentiated ASMCs exposed to proliferating stimuli, exhibit decreased expression of contractile phenotype marker proteins [7]. However, prolonged incubation of ASMCs with gallamine, up to 15 days, did not affect the percentage of differentiated ASMCs that express the contractile phenotype marker proteins, α-actin, MHC and desmin. On the contrary, cell incubation with 10% FBS, caused a significant decrease of the contractile markers' immunoreactivity (Fig. 6).

Discussion

Anticholinergics are commonly used alone or as combined therapy for the treatment of bronchoconstriction, manifested in airway diseases, such as uncontrolled asthma or COPD [17,18]. In addition to the well-established effect of anticholinergic drugs on airway responsiveness, their potential effect on airway remodeling is also of particular interest, although less well studied and likely more complex. Along these lines, there are few reports demonstrating the ability of muscarinic antagonists to modulate intracellular signals [23] and affect cholinergic receptors density on the cell surface [24]. For this purpose, we studied the effect of the muscarinic receptor modulator gallamine [25,26] and the non-selective muscarinic receptor antagonist atropine on ASMC proliferation of 24 h serum-starved cells. We further investigated the mitogenic effect of gallamine on 7 days serum-starved ASMCs as prolonged serum starvation shifts ASMC to a contractile phenotype [7].

Our results show that gallamine had a mitogenic effect on both 24 h (Fig. 1) and 7 days (Fig. 5) serum-starved rabbit ASMCs. On 24 h starved cells the effect of gallamine appears to be dose-dependent and time independent. Our experimental procedure, does not allow to directly determine the binding sites of gallamine on the ASMC cytoplasmic membrane. However, we believe that the mitogenic effect of gallamine on ASMCs is mediated *via* binding on muscarinic receptors. This kind of data interpretation is based on previously published data showing that gallamine, is as allosteric modulator, interacting specifically with muscarinic receptors [23,27,28]. Indicatively, it has been found that gallamine, in the absence of an agonist, alters intracellular signaling molecules (AMP, IPs) in CHO cells expressing the M_2 or M_4 muscarinic receptor subtype the way carbachol does and that, this effect was

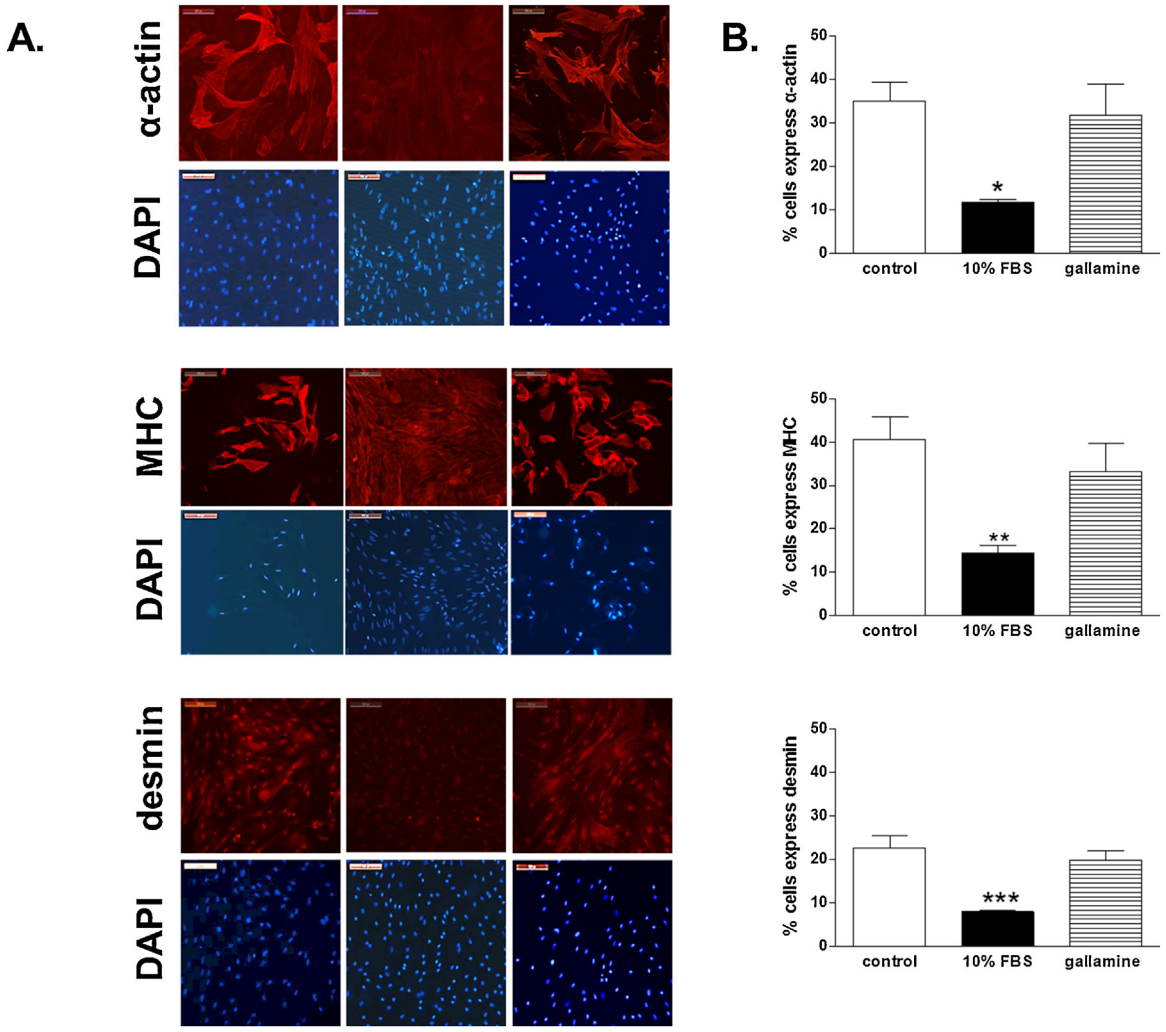

Fig. 6. Gallamine does not affect the expression of ASMC contractile marker proteins. ASMCs were incubated for 15 days in serum free medium (control) or in the presence of 10% FBS or gallamine (10 mM). (A) Indirect immunofluorescence analysis of smooth muscle α-actin (upper panel), MHC (middle panel) and desmin (lower panel). (B) Quantitative analysis of fluorescence microscope images showing the percentage of cells that express smooth muscle α-actin (upper panel), MHC (middle panel) and desmin (lower panel). Data represent the mean ± SEM of cells ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control).

not observed in CHO cells that have not been transfected with the gene for any of the subtypes of muscarinic receptors [23].

Moreover, previous studies have shown that gallamine acts as a classical muscarinic antagonist, with high affinity to M_2 receptors, at low concentrations, while at high concentrations it displays an allosteric interaction with muscarinic receptors in general. Based on the fact that, the mitogenic effect of gallamine was statistically significant at high concentrations (>1 μM), our results suggest that the mitogenic effect of gallamine on ASMCs is mediated *via* binding to the allosteric site rather than via muscarinic receptor antagonism. This is not surprising, as the ability of allosteric modulators to mediate receptor activation on their own, in the absence of an orthosteric agonist, has been shown before [23,29]. Accordingly, we show in this study that, in analogy to muscarinic agonists [7], gallamine-induced proliferation is mediated *via* MAPK and PI3K pathways (Fig. 4) as the PI3K signaling pathway inhibitor, LY294002, as well as the MAPK signaling pathway inhibitor PD98059, decreased gallamine-induced proliferation.

Moreover, gallamine (10 mM) stimulated the proliferation of 7 days starved ASMCs, but did not alter the mitogenic effect of the muscarinic agonist acetylcholine (Fig. 5), suggesting that probably both agents exert their effects by stimulating the same signaling pathways, probably by engaging on distinct sites of the muscarinic receptors. On the contrary, acetylcholine reduced the gallamine-induced proliferation of ASMCs (Fig. 5B). Indeed, although muscarinic agonists are primarily considered as mitogenic, an anti-mitogenic effect has also been documented [16], as M_2 receptors stimulation can either reduce or increase intracellular cAMP *via* activation of Gi or Gs proteins respectively [30], depending on the density of activated receptors and agonist concentration [31]. In addition, long lasting cell treatment with gallamine has been reported to up-regulate M_2 receptors [24]. It is therefore possible, that acetylcholine may decrease gallamine-induced ASMC proliferation *via* an increase in cAMP production [32].

When we used FBS as a mitogenic stimulus, gallamine did not alter the proliferation induced by 10% FBS in 24 h (Fig. 3A) serum starved cells. On the contrary, gallamine (10 mM) reduced ASMC proliferation induced by incubation in the presence of 1% FBS (Fig. 3B). This reduction cannot be attributed to a decrease of cell viability in the presence of gallamine, as no further cell number reduction was observed after ASMCs treatment for 6 days in the presence of 1% FBS and gallamine (10 mM). Even though a similar dual, proliferative and antiproliferative effect induced by muscarinic agonists (4-DAMP and AF-DX-116) has been shown in rat ASMCs [16], it cannot be directly interpreted from our results. Most

likely, in the presence of 10% FBS ASMCs exhibit a higher proliferation rate and a lower muscarinic receptor concentration, compared to cells cultured in the presence of 1% FBS [33]. On the other hand, ASMC proliferation might be fine-tuned by a cross talk between M_2 and M_3 receptors, as has been reported in CHO cells regarding intracellular signals and specifically ERK activation [34,35].

The mitogenic effect of gallamine is also exerted by other muscarinic antagonists. We found that, similarly to gallamine, the non-selective muscarinic antagonist atropine induces ASMC proliferation in a dose dependent manner (Fig. 2). To our knowledge atropine is not an allosteric modulator of muscarinic receptors, but it may act as an inverse agonist [19,24,36]. G protein coupled receptors have the potential to be active even in the absence of an agonist and trigger production of second messengers [36]. Atropine, in the absence of any agonist, has been shown to increase the intracellular levels of cyclic nucleotides in bovine tracheal smooth muscle strips [37]. The pathways involved in the mitogenic effect of atropine on ASMCs need further investigation.

Furthermore, ASMCs have been reported to express choline acetyltransferase [13] and therefore being able of releasing acetylcholine. This means that, in our system, gallamine could have an antagonistic effect to the endogenous acetylcholine actions. Though this explanation seems less likely, given our results showed that gallamine does not alter the mitogenic effect of externally applied acetylcholine (Fig. 5), one cannot exclude the possibility that at low concentrations, endogenous acetylcholine preferentially acts on one muscarinic receptor subtype through which gallamine exerts its proliferative effect, while at concentrations similar to the ones we exogenously supplied, acetylcholine acts on another muscarinic receptor subtype through which it exerts it's proliferative effects. This could also explain why acetylcholine has a proliferative effect on ASMCs only when phenotype shifting has occurred; that is M_3 receptor density increased [7].

The identification of the muscarinic receptor subtypes engaged in antagonist-induced mitogenic effect was not feasible, as gallamine at high concentrations may modulate all types of muscarinic receptors, while atropine is a bona fide non-selective muscarinic antagonist. Earlier studies have described the presence of mainly M_2, M_3, and M_4 muscarinic receptors subtypes on rabbit ASMCs [6]. Although M_3 or M_2 receptors are considered to be mainly responsible for ASM cell proliferation, we cannot rule out the possibility that a mitogenic effect could be mediated *via* M_4 receptors, in analogy to results obtained from studies in other cell types [38,39].

The increase in proliferation induced by gallamine was not accompanied by a decrease in expression of proteins that are markers of the contractile cell phenotype, such as α-actin, MHC and desmin (Fig. 6). Previous studies have shown that long term exposure to muscarinic agonists affects the phenotype obtained by ASMCs as a response to the mitogenic stimuli [7]. The lack of such effect implies that even though gallamine is capable of increasing cell proliferation it does not have the ability to cause a phenotype shift, probably due to the type of muscarinic receptors present on these cells and/or the allosteric way it affects them. These observations could explain the transient effect on ASMC proliferation as well.

Conclusions

This study demonstrates that muscarinic receptor modulators can transiently induce proliferation in ASMCs, without affecting the expression of contractile proteins. This effect is mediated by the PI3K and MAPK signaling pathways and is not affected by the cell phenotype or the presence of other mitogenic stimuli.

Conflict of interest

The authors declare that there is no conflict of interest and that they did not receive any financial aid.

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