Chapter 7

Muscarinic M₃-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle

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Abstract
Muscarinic receptor agonists have been considered to act synergistically in combination with growth factors on airway smooth muscle (ASM) growth. Characterization of the proliferative responses and of the receptor subtype(s) involved has not yet been studied. Therefore, we investigated mitogenesis induced by stimulation of muscarinic receptors, alone and in combination with stimulation by platelet-derived growth factor (PDGF). For this purpose, [3H]thymidine-incorporation was measured at different culture stages in bovine tracheal smooth muscle (BTSM) cells. Functional muscarinic M3-receptors, as measured by formation of inositol phosphates, were present in unpassaged cells, but were lacking in passage 2 cells. Methacholine (10 µM) by itself was not able to induce a proliferative response in both cell culture stages. However, methacholine interacted synergistically with PDGF in a dose-dependent fashion (0.1-10 µM), but only in cells having functional muscarinic M3-receptors. This synergism could be suppressed significantly by the selective M3-receptor antagonists DAU5884 (0.1 µM) and 4-DAMP (10 nM), but not at all by the M2-subtype selective antagonist gallamine (10 µM). These results show that methacholine potentiates mitogenesis induced by PDGF solely through stimulation of muscarinic M3-receptors in BTSM cells.

Introduction
Airway smooth muscle (ASM) expresses muscarinic receptors of the M2- and the M3-subtype. The M3-subtype is responsible for contraction, largely through Gq-mediated activation of phosphoinositide turnover and subsequent Ca2+ mobilization [1]. The role of the majority of M2-receptors in ASM is still unclear, however. In bovine tracheal smooth muscle (BTSM), a role for the M2-receptor in functional antagonism of β-adrenergic responses through inhibition of adenylyl cyclase has been suggested. This was observed only after substantial 4-DAMP mustard-induced alkylation of the M3-receptor population [2]; under normal conditions, M2-receptors are not interfering with the isoprenaline-induced relaxation of cholinergic tone as demonstrated by the lack of effect of selective M2-receptor blockade [3]. Recently, however, a role for M2-receptors in Ca2+-sensitization and cytoskeletal reorganization has been proposed [4-6]. Furthermore, M2-receptors may stimulate non-selective cation channels through Gi/G(o)-proteins, resulting in a rise in [Ca2+]i [7].

Muscarinic receptor agonists have been reported to be mitogenic for human ASM cells, though at most modestly, and to respond synergistically in combination with growth factors [8;9]. Although carbachol-induced mitogenesis has been reported to be pertussis toxin (PTX)-sensitive [8;10], suggesting a role for the Gq-protein-coupled muscarinic M2-receptor, measurements were carried out using human ASM cells in culture, which are known to have a relatively small functional M2-receptor population compared to non-cultured cells. This loss in receptor function is far less profound for the M3-receptor subtype [11]. A role for the M3-subtype in proliferation can therefore not be ruled out, nor can the putative relevance of the M2-subtype in ASM proliferation be properly estimated because of the diminished presence of muscarinic M3-receptors. Moreover, although PTX was found to decrease the
carbachol-induced mitogenic response, proliferation to all applied stimuli was reduced to a similar extent by treatment with PTX [8]. Therefore, it may not be appropriate to conclude that muscarinic receptor stimulation-induced mitogenic responses are M2-receptor mediated.

Theoretically, both M2 and M3-receptors could account for the mitogenic contribution of muscarinic receptor stimulation. The p42/p44 mitogen activated protein kinase (MAPK)-pathway is generally associated with proliferation and is known to be involved in the proliferative responses to various mitogens in BTSM [12-15]. Muscarinic M3-receptor-stimulation induces a considerable rise in \([\text{Ca}^{2+}]\), which may lead to the activation of p42/p44 MAPK through Pyk2 and Ras-dependent mechanisms [16]. Moreover, the M3-receptor subtype activates protein kinase C (PKC), which may lead to p42/p44 MAPK-activity through PKC\(\alpha\)-mediated phosphorylation of Raf-1 [17]. In support of this hypothesis, CHO-cells transfected with the wild-type M3-receptor subtype, demonstrated activation of the p42/p44 MAPK pathway induced by carbachol [18]. This M3-receptor mediated p42/p44 MAPK-activity was inhibited almost completely using the PKC-inhibitor Ro-318220. Moreover, stimulation with the PKC-activator PDBu could partially mimick this response, suggestive of a significant role for PKC in the response [18;19].

G-proteins, however, activated by muscarinic M2-receptors, may also activate the p42/p44 MAPK-pathway through either \(G_{\alpha}\) [20] or \(G_{\beta\gamma}\)-dependent [21] mechanisms. In canine colonic smooth muscle, it has been demonstrated that M2- rather than M3-receptors are responsible for p42/p44 MAPK-activation [22]. Moreover, it has been demonstrated that M2-receptors activate the non-receptor tyrosine kinase Src in the same tissue [23], which acts as a key intermediate in tyrosine kinase signaling. Also in CHO-cells, transfected with the wild-type M2-receptor, methacholine-induced p42/p44 MAPK-activation has been reported [24].

Proliferative responses following selective M2- or M3-muscarinic receptor stimulation in airway smooth muscle have not yet been described. In the present study, we investigated their putative involvement in BTSM cells. Since muscarinic M3-receptors have been reported to lose their function rapidly in culture [11], we used both passaged and unpassaged BTSM cells. In order to gain insight in the receptor subtype(s) involved in methacholine-induced mitogenic responses, we studied the effects of subtype-selective receptor blockade. It was demonstrated that mitogenic responses to muscarinic receptor stimulation alone were absent. However, the mitogenic responses to platelet-derived growth factor (PDGF) were augmented by methacholine, which was solely mediated by the M3-receptor subtype.

**Methods**

**Isolation of bovine tracheal smooth muscle cells**

Bovine tracheae were obtained from local slaughterhouses and transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, MgSO\(_4\) 1.18, CaCl\(_2\) 2.50, NaH\(_2\)PO\(_4\) 1.28, NaHCO\(_3\) 25.00 and
glucose 5.50, pregassed with 5 % CO₂ and 95 % O₂; pH 7.4. After dissection of the smooth muscle layer and removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 300 µm and three times at a setting of 100 µm. Tissue particles were washed two times with Dulbecco’s Modification of Eagle’s Medium (DMEM), supplemented with NaHCO₃ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml) and 0.5 % Fetal Bovine Serum (FBS). Enzymatic digestion was performed using the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and Soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37°C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over 50 µm gauze, cells were washed three times in DMEM, supplemented as above, containing 10 % FBS.

Cell culture
After isolation, BTSM cells were either used directly for experiments (unpassaged cells) or seeded in 25 cm² culture flasks at a density of 1x10⁶ cells/ml for further culturing. Cultured cells were kept viable in medium containing 10 % FBS at 37°C in a humidified 5 % CO₂-incubator. Medium was refreshed every 48-72 h. Cell cultures were allowed to grow and, upon confluency, were passaged further at a 1:2 split ratio, by means of trypsinization. Cultured cells were used for experiments in passage 2.

[^3]HThymidine-incorporation
BTSM cells were plated in 24 well cluster plates at a density of 30,000 cells per well and were allowed to attach overnight in 10 % FBS containing medium at 37°C in a humidified 5 % CO₂-incubator. Cells were washed two times with sterile phosphate buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH₂PO₄, 1.4; Na₂HPO₄·2H₂O, 8.1; pH 7.4) and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 72 h. Cells were then washed with PBS and stimulated with mitogens in FBS-free medium for 28 h, the last 24 h in the presence of [^3]Hthymidine (0.25 µCi/ml), followed by two washes with PBS at room temperature and one with ice-cold 5 % trichloroacetic acid (TCA). Cells were treated with this TCA-solution on ice for 30 min; subsequently the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated[^3]Hthymidine was quantified by liquid-scintillation counting.

Accumulation of[^3]H-inositol phosphates
BTSM cells were plated after isolation or after passage 2 in 6 well cluster plates at a density of 1x10⁵ cells/well. After attachment overnight in medium containing 10 % FBS at 37°C in a humidified 5 % CO₂-incubator, cells were washed twice in sterile PBS and treated with serum-free medium for 72 h containing apo-transferrin (5 µg/ml), ascorbate (100 µM), insulin (1 µM) and[^3]H-inositol (2 µCi/ml). Next, cells were washed twice with Ringer buffer containing (in mM) NaCl 125.0, KCl 6.0, MgCl₂ 2.5, CaCl₂ 1.2, NaH₂PO₄ 1.2, HEPES 25.0 and glucose 11.0, pH 7.4. After a 15 min
incubation period in the same buffer, supplemented with 5 mM LiCl, cells were stimulated with methacholine in varying concentrations for another 30 min. Reactions were terminated by replacing the Ringer buffer for 1 ml of a methanol:0.12 mM HCl mixture (1:1 v/v), which had been previously kept at –20 °C and cells were allowed to lyse for another 30 min at –20 °C. 800 µl of the lysate was neutralized to pH = 7 using 3 ml buffer (composition 25 mM Tris / 0.5 M NaOH / H2O 10:1:30 v/v/v) and [3H]inositol phosphates were finally separated from free [3H]inositol using Dowex-AG 1X8 anion exchange chromatography as described by Hoiting et al. [25].

Data analysis
All data represent means ± s.e.mean from n separate experiments. The statistical significance of differences between data was determined by the Student’s t-test for paired observations or one-way ANOVA where appropriate. Differences were considered to be statistically significant when P < 0.05.

Materials
Dulbecco’s modification of Eagle’s Medium (DMEM) and methacholine hydrochloride were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Fetal bovine serum, NaHCO3 solution (7.5 %), HEPES solution (1 M), sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml / 5000 µg/ml), amphotericin B solution (250 µg/ml) (Fungizone) and trypsin were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Platelet-derived growth factor AB (PDGF-AB, human recombinant), insulin (from bovine pancreas), apotransferrin (human), soybean trypsin inhibitor and gallamine triethiodide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). DAU5884 was a kind gift of Dr H. N. Doods (Dr Karl Thomae GmbH, Biberach, Germany) and 4-DAMP methobromide was kindly provided by Dr R. B. Barlow, Bristol, UK. [Methyl-3H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.). [3H]myo-inositol (specific activity 59.9 Ci/mmol) was obtained from NEN Life Sciences Products (Boston, MA, USA). Papain and collagenase P were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

Results
Methacholine-induced accumulation of inositol phosphates in unpassaged and passage 2 bovine tracheal smooth muscle cells.
BTSM cells treated for 1 day with 10 % FBS, followed by 3 days with serum-deprived medium (unpassaged BTSM cells) responded to methacholine with an increase in the accumulation of inositol phosphates (maximal effect (Emax) = 348 ± 43 % of basal, sensitivity (pEC50) = 5.61 ± 0.21). In contrast, in cells cultured up to passage 2 no dose-dependent effects of methacholine could be determined. In this condition, basal formation of inositol phosphates remained unchanged for all concentrations of methacholine applied (average 98 ± 6 %, Figure 7.1).
Effects of methacholine, alone and in combination with PDGF on [³H]thymidine-incorporation in unpassaged and passage 2 bovine tracheal smooth muscle cells.

Stimulation of passage 2 BTSM cells with PDGF (10 ng/ml) induced an increase in [³H]thymidine-incorporation to 255 ± 46 % of basal. Methacholine (10 µM) did not induce any proliferative response (91 ± 14 % of basal) and did not potentiate the proliferative response to PDGF either (295 ± 59 %, Figure 7.2A). In unpassaged BTSM cells, methacholine (10 µM) was unable to produce a proliferative response by itself (114 ± 14 %). Interestingly, the PDGF-induced increase in incorporated thymidine was significantly potentiated in these cells (246 ± 33 % and 371 ± 59 % for PDGF and PDGF + methacholine-treated cells, respectively, P < 0.05. Note that passage number did not influence the PDGF-response (Figure 7.2B).

Figure 7.1 Methacholine-induced inositol phosphates (IP₃)-accumulation in unpassaged (open circles) and passage 2 (closed circles) BTSM cells. Data represent means ± s.e.mean from 3 experiments each performed in triplicate.

Figure 7.2 [³H]thymidine-incorporation induced by 10 µM methacholine (MCh), 10 ng/ml PDGF and the combination in both passaged (passage 2) BTSM cells (panel A) and unpassaged BTSM cells (panel B). Data represent means ± s.e.mean from 4 (passaged) and 6 (unpassaged) experiments each performed in triplicate. * P < 0.05 compared to basal; † P < 0.05 compared to PDGF.
The effects of methacholine appeared to be concentration-dependent. Although at concentrations of 0.1-10 µM methacholine was unable to induce a proliferative response by itself, the agonist raised the $E_{\text{max}}$ and reduced the $EC_{50}$ of PDGF in a concentration-dependent fashion, which was most pronounced at 10 µM methacholine ($E_{\text{max}} = 326 \pm 45 \%$ and $421 \pm 46 \%$, $P < 0.001$ and $EC_{50} = 4.8 \pm 0.8$ and $3.0 \pm 0.5$ ng/ml, $P < 0.05$ for PDGF and PDGF + 10 µM methacholine, respectively, Figure 7.3).

**Effects of subtype-selective antagonists on methacholine and PDGF-induced synergism in [³H]thymidine-incorporation**

To establish the muscarinic receptor subtype(s) involved in the mitogenic responses to methacholine, we measured the inhibitory effects of selective receptor antagonists (DAU5884, 4-DAMP and gallamine) on the methacholine-induced potentiation of the proliferative response to PDGF.

![Figure 7.3](image)

**Figure 7.3** Dose-dependent [³H]thymidine-incorporation in response to PDGF in unpassaged BTSM cells in the absence (open circles) or presence of 0.1 µM (filled circles, panel A), 1 µM (filled squares, panel B) and 10 µM methacholine (filled triangles, panel C). Data represent means ± s.e.mean from 6 (0.1 and 1 µM methacholine) and 12 (10 µM methacholine) experiments, each performed in triplicate. * $P < 0.05$; *** $P < 0.001$ compared to control.

Synergism was calculated as the methacholine-induced increase in the PDGF response and expressed relative to a control response (10 ng/ml PDGF). As shown in Figure 7.4A, synergism was dependent on the dose of methacholine applied. Maximal methacholine-induced synergism averaged 8.9 ± 10.6, 36.0 ± 15.9 and 55.3 ± 11.9 % for 0.1, 1 and 10 µM methacholine, respectively.
The potentiation induced by 10 µM methacholine was almost completely suppressed by DAU5884 (0.1 µM) (Figure 7.4B). The concentration of DAU 5884 applied results in an almost complete blockade of the M₃-receptor, with minor effects on the M₂-receptor (Table 7.1). Another M₃-receptor selective antagonist, 4-DAMP (10 nM), with a lower fractional M₃-receptor occupancy (Table 7.1) also inhibited the methacholine-induced synergism, though not completely (Figure 7.4B). In contrast, the M₂-receptor selective antagonist gallamine (10 µM, Table 7.1) did not alter the synergistic response (Figure 4B), which was virtually identical to control (compare with Figure 7.4A, open symbols).

![Figure 7.4](image)

**Figure 7.4** Panel A: Synergism in [³H]thymidine-incorporation in unpassaged BTSM cells, induced by 0.1 µM (triangles), 1 µM (inverted triangles) and 10 µM (open circles) methacholine in combination with PDGF. Data represent means ± s.e.mean from 6-12 experiments each performed in triplicate. Panel B: Synergism in [³H]thymidine-incorporation in unpassaged BTSM cells, induced by 10 µM methacholine in combination with PDGF in the presence of DAU5884 0.1 µM (diamonds), 10 nM 4-DAMP (filled circles) or 10 µM gallamine (squares). Data represent means ± s.e.mean from 4-6 experiments each performed in triplicate. * P < 0.05 compared to absence of antagonist.

**Table 7.1** Fractional occupancy of muscarine M₂- and M₃-receptors by gallamine, DAU 5884 and 4-DAMP.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Fractional M₂-receptor occupancy (%)</th>
<th>Fractional M₃-receptor occupancy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gallamine, 10 µM</td>
<td>98.8</td>
<td>8.4</td>
</tr>
<tr>
<td>DAU 5884, 0.1 µM</td>
<td>26.2</td>
<td>99.1</td>
</tr>
<tr>
<td>4-DAMP, 10 nM</td>
<td>35.0</td>
<td>95.4</td>
</tr>
</tbody>
</table>

Estimated values for fractional occupancy were obtained using affinity data reported by Roffel et al. [26;27]
Discussion
Cultured airway smooth muscle cells undergo a variety of changes in protein expression and function as a consequence of modulation towards a synthetic phenotype. This phenotypic modulation is known to diminish the contractile properties of the airway smooth muscle cell; in contrast, their synthetic and proliferative capacities increase [28]. Plasticity in receptor-protein expression is one of the consequences of phenotypic modulation, which may both lead to down-regulation (e.g. muscarinic M\textsubscript{3}-receptors in human ASM) and up-regulation of receptor proteins (e.g. PDGF-\(\beta\) receptors in porcine uterus) [29;30]. As regards muscarinic receptors in cultured ASM cells, the M\textsubscript{3}-subtype is known to be downregulated rapidly upon culturing, whereas the M\textsubscript{2}-receptor function is not markedly altered for up to passage 10 in human ASM cells [11]. Therefore it is important to characterize the functional presence of the M\textsubscript{3}-receptor under different cell culture conditions, particularly since our aim was to study the relative involvement of M\textsubscript{2} and M\textsubscript{3}-receptors in mitogenic responses.

In the unpassaged condition, M\textsubscript{3}-receptor function, assessed by methacholine-induced increase in inositol phosphates formation, was comparable to earlier observations in freshly isolated BTSM cells [25]. It is of importance to note that the M\textsubscript{3}-receptor is the only receptor mediating inositol phosphates formation in BTSM, as established using selective muscarinic receptor antagonists [31]. In contrast to the unpassaged condition, the M\textsubscript{3}-receptor was no longer functional in cells that were passaged twice. It has been shown that passaged cultured canine ASM cells do not functionally express M\textsubscript{3}-receptors either, whereas subsequent prolonged deprivation of serum re-induces functional coupling of the M\textsubscript{3}-receptor, selectively for a subset of elongated contractile cells [32]. These elongated cells shorten by > 70% of their original length in response to acetylcholine and express M\textsubscript{3}-receptors on the outer membrane, whereas serum-fed cells demonstrate a perinuclear distribution of M\textsubscript{3}-receptors that are not functionally coupled to inositol phosphates production [33]. This shows that cell surface coupled M\textsubscript{3}-receptors are reversibly lost upon transition to the synthetic phenotype. Most likely, this plastic behaviour in M\textsubscript{3}-expression is the basis behind the differences in functional M\textsubscript{3}-responses in the different cell culture stages. Recently, it was shown that AP-2\(\alpha\) may act as an important transcriptional regulator in this process: the M\textsubscript{3}-receptor gene contains 8 AP-2 consensus binding motifs and AP-2\(\alpha\) is known to be upregulated upon serum withdrawal [34].

In passaged human ASM cells, it has been found that the inositol phosphates response to carbachol is still present, although decreased. The loss of functional M\textsubscript{3}-receptors in human ASM appeared to be largely determined by post-transcriptional regulation, not by decreased mRNA expression [11]. This process might be even more active in BTSM cells in view of the total lack of response.

Interestingly, the same culture condition that maintained functional M\textsubscript{3}-expression, ensured a synergistic mitogenic response for methacholine and PDGF. In passaged (passage 2) cells, however, this synergism in mitogenesis was absent, as was
functional M₃-receptor expression. These differences suggest a role for the M₃-receptor in the proliferative potentiation induced by methacholine. Indeed, selective blockade of the M₃-receptor by DAU5884 or 4-DAMP resulted in suppression of this synergistic response. The suppression was not as profound for 4-DAMP as it was for DAU5884. However, DAU5884 was used in a concentration that results in 0.9 % of the M₃-receptor fraction available for stimulation by methacholine, whereas in case of 4-DAMP, a higher fraction (4.6 %) of M₃-receptors remained unoccupied. Remarkably, selective blockade (98.8 %) of the M₂-receptor by gallamine was totally ineffective. Hence, the M₃-receptor apparently is the only subtype involved in the regulation of the mitogenic responses by methacholine in unpassaged BTSM cells. Since M₃-expression in cultured airway myocytes appears not to be homogeneous [32;33], this could indicate that muscarinic agonist-induced growth synergy is mediated by a particular subset of these cells. In 1321N1 human astrocytoma cells acetylcholine did also induce proliferation via M₃-receptors, despite the presence of M₂ and M₅-subtypes [35].

These findings raise the question why this synergistic response is absent in passaged BTSM cells, but present in passaged human ASM cells, as described previously [9]. This difference may be the result of species-differences in M₃-receptor expression: although diminished, the presence of a functional M₃-receptor population in cultured human ASM has been reported on several occasions, having a response of approximately 35 % of that induced by histamine, as determined by the formation of inositol phosphates [11;36]. Furthermore, a small, but not absent population of functional M₃-receptors is consistent with the finding that carbachol is relatively weak in inducing proliferation synergy in human ASM cells when compared to other G protein coupled agonists [8;9].

Methacholine did not produce a proliferative response by itself, which is in line with observations by others, showing no [9] or a relatively small [8] increase in proliferation of ASM induced by muscarinic agonists. However, muscarinic receptor stimulation may play an important modulatory role. Muscarinic receptor stimulation was mitogenic only in combination with other mitogens, like growth factors. The combined response induced by methacholine (10 µM) and a concentration of PDGF (1 ng/ml), unable to induce proliferation by themselves, resulted in approximately 45 % of the maximal PDGF-induced response under control conditions. This would imply a threshold either in the activation of transduction cascades or in the response of the cell to transductional activation.

The modulatory role may become of physiological relevance particularly in an environment, in which growth factors are abundant, for instance due to secretion by inflammatory cells. Therefore, endogenous acetylcholine may contribute to the pathophysiology of inflammatory airway diseases, in which an increase in smooth muscle mass leads to airflow obstruction such as chronic asthma [37].

On a molecular level, M₃-mediated mitogenic responses are in agreement with biochemical studies, showing that G₃-coupled muscarinic receptors may couple to
pathways known to be involved in transcriptional regulation [38], such as the p42/p44 MAPK-pathway [24], the PI-3-kinase/PKB-pathway [39], and stress-activated members of the MAPK-superfamily, like the c-Jun N-terminal kinase (JNK)-pathway [24] and the p38-MAPK-pathway [40]. In addition, coupling to $G_{12/13}^\alpha$ proteins may be responsible for the observed effects. Although less well studied, $G_{12/13}^\alpha$ subunits are known to be involved in cellular growth and to potently activate JNK, whereas ERK-activity may both be inhibited or stimulated, dependent on the cell type [41].

The more challenging question is why $M_2$-receptors do not seem to be relevant, since in theory, $M_2$-receptors may couple to the same pathways [24;42;43]. Moreover, other $G_i$ coupled-stimuli have been shown to respond synergistically in combination with growth factors in human ASM cells [8]. Perhaps other transductional pathways are involved in the muscarinic receptor-mediated mitogenic responses. Though, differences in the kinetics of activation may also account for the unexpected observation. In CHO-cells, transfected with either human wild-type $M_2$- or $M_3$-receptors, it was found that both the $M_2$- and the $M_3$-transfected cells mediated p42/p44 MAPK- and JNK-activation; however, $M_2$-receptor mediated responses were transient, whereas $M_3$-receptor responses were sustained [24]. This difference may be of critical importance, since sustained p42/p44 MAPK/ERK-activity determines the proliferative responses in human [44] and bovine ASM cells [14]. Since little is known about the quantitative contribution of the pathways mentioned in regulating cross-talk between G-protein coupled receptors and tyrosine kinase coupled receptors, the absence of a $M_2$-mediated mitogenic response might perhaps not be generalized.

In conclusion, in BTSM cells methacholine does not induce mitogenesis by itself, but potentiates PDGF induced proliferation. This was dependent on the presence of functional $M_3$-receptors as controlled by the cell culture conditions applied. This synergism could be abolished by selective $M_3$-receptor antagonists, like DAU5884 and 4-DAMP, but not by the $M_2$-subtype selective antagonist gallamine. These results show that methacholine potentiates mitogenesis induced by PDGF through stimulation of $M_3$-receptors in BTSM cells.

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References


