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Acetylcholine enhances keratocyte proliferation through muscarinic receptor activation



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ABSTRACT

Acetylcholine (ACh), a classical neurotransmitter, has been shown to be present in various non-neuronal cells, including cells of the eye, such as corneal epithelium and endothelium, and to have widespread physiological effects such as cytoskeleton reorganization, cellular proliferation, differentiation, and apoptosis. The aim of this study was to investigate the effect of ACh on corneal keratocyte proliferation, and the underlying mechanisms, in order to explore its possible effect in corneal wound healing. Primary culture of human keratocytes was established from donated corneas. Cell viability and fraction of proliferating cells were detected by MTS assay and BrdU incorporation ELISA, respectively. Expression of proliferative markers, PCNA and Ki-67, was detected by western blot and immunocytochemistry. Activation of the MAPK/Erk signaling pathway and its involvement in ACh-enhanced proliferation was determined by western blot analysis, MTS, and BrdU ELISA. We found that ACh enhanced keratocyte proliferation even at low concentrations. Stimulation of proliferation was mediated through activation of muscarinic ACh receptors (mAChRs). Western blot analysis revealed that ACh stimulation of keratocytes upregulated the expression of PCNA and Ki-67, and Ki-67 immunocytochemistry showed that ACh-treated cells were in an active phase of the cell cycle. ACh activated MAPK signaling, and this step was crucial for the ACh-enhanced proliferation, as inhibition of the MAPK pathway resulted in ACh having no proliferative effect. In conclusion, ACh enhances keratocyte proliferation and might thus play a role in proper corneal wound healing.

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1. Introduction

Acetylcholine (ACh) is regarded as a classical neurotransmitter well known for acting in the central and peripheral nervous system, and to be present in both prokaryotes and eukaryotes [1–3]. However, in recent years, there have been extensive evidence demonstrating a non-neuronal cholinergic system, in which ACh and/or its synthesizing enzyme, choline acetyltransferase (ChAT), have been found to be present in non-neuronal human cells, such as keratinocytes [1,4], epithelial [5] and endothelial [6] cells, tendons [7], and various cells of the immune system [3]. ACh has been shown to have widespread physiological effects such as cytoskeleton reorganization, cellular proliferation, differentiation, and apoptosis [1,3,8,9]. Most cell types not innervated by cholinergic neurons still express two types of ACh receptors [10]: nicotinic (nAChRs), which are ion channels, or G protein-coupled muscarinic receptors (mAChRs). Activation of these receptors has been linked to activation of mitogen-activated protein kinase (MAPK)-dependent pathways [9,11,12], in which phosphorylation of Erk1/2 leads to DNA synthesis and cell proliferation [13].

The cornea is the transparent front part of the eye and is composed of three distinct layers: an outermost epithelial layer, a middle, transparent stromal layer, and an innermost endothelial layer. The stroma accounts for up to 90% of the corneal thickness and it is made of collagen fibrils in between which the quiescent cells of the stroma, the keratocytes, reside. The main role of these cells is maintenance and repair of the stroma [14–16]. ACh and ACh receptors have been studied in the cornea; however, most of the studies have been conducted in corneal epithelium and endothelium. Corneal epithelium has been shown to contain high concentrations of ACh and its synthesizing enzymes ChAT, and both corneal epithelium and endothelium express mAChRs [17]. There has been less attention to expression of nAChRs in the cornea. However, a recent study has shown that corneal epithelium expresses several nAChRs, including both $\alpha 7$ and $\alpha 9$ homomeric nAChRs and predominantly the $\alpha 3\beta 2 \pm \alpha 5$ subtype of heteromeric nAChRs [18]. The role of ACh in the cornea remains unclear, but data suggest that ACh plays an important role in epithelial regrowth after corneal injury and that it promotes chemotaxis [18], therefore facilitating healing of the injured cornea. Recent findings in our lab have shown that *in vitro* cultured human keratocytes secrete ACh and express mAChRs of subtypes M_1 , M_3 , M_4 , and M_5 (unpublished data). Nevertheless, the knowledge on the ACh system in the corneal stroma, and its keratocytes, is still

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scarce, and a deeper understanding of the potential role of ACh in corneal wound healing requires studies on the stromal components. Therefore, in the present study, we evaluate the role ACh in keratocyte proliferation and thus its possible role in corneal wound healing.

2. Materials and methods

2.1. Collection of human corneas

Healthy human corneas from deceased individuals who had chosen, when alive, to donate their corneas post-mortem for transplantation or research, according to Swedish law, were kept in a corneal biobank at the University Hospital of Umeå, Sweden. If these healthy donated corneas were not used for transplantation after their collection, they were delivered to the laboratory for research purpose. If corneas were used for transplantation, some or all of the transplantation graft leftovers were retrieved for study purpose: the healthy donor peripheral part or the healthy donor anterior or posterior central lamella. The study was vetted by the Regional Ethical Review Board in Umeå (2010-373-31 M) without objections. The study was performed according to the principles of the Declaration of Helsinki.

2.2. Chemicals and reagents

Acetylcholine, mecamlamine, atropine, and collagenase were purchased from Sigma–Aldrich (Saint Louis, MO, USA). PD98059 was obtained from Calbiochem (San Diego, CA, USA). Antibodies against β -actin, p-Erk1/2 (Thr202/Tyr204), and PCNA were purchased from Cell Signaling (Danvers, MA, USA). Ki-67 antibody was obtained from Millipore (Billerica, MA, USA). Cell viability assay was purchased from Promega (Fitchburg, WI, USA). BrdU incorporation ELISA was purchased from Roche (Basel, Switzerland). DMEM/F-12 + GlutaMAX™ medium, penicillin-streptomycin, FBS, and Trypsin-EDTA were obtained from Life Technologies (Carlsbad, CA, USA).

2.3. Isolation and primary culture of human corneal stromal cells

Primary culture of human keratocytes was established by first scraping off any remaining epithelial and endothelial cells from a donated cornea, and then separating and mincing central and peripheral parts with a scalpel. Samples were then digested in 2 mg/ml collagenase diluted in DMEM/F-12 + GlutaMAX™ medium overnight at 37 °C. The samples were then transferred to DMEM / F-12 GlutaMAX™ medium supplemented with 2% FBS and 1% penicillin-streptomycin and cultured at 37 °C with 5% CO₂. Medium was replaced every second to third day until the cells reached confluence. Confluent cells were detached with 0.05% Trypsin-EDTA and split in a 1:2 ratio. Cells from the central cornea in passages 4 to 5 were used for experiments. DMEM/F-12 GlutaMAX™ medium supplemented with 0.1% FBS was used both for seeding cells and performing experiments. In total, 12 corneas were used for experiments. The corneas were assessed individually.

2.4. Cell viability assay

The effect of ACh on the viability and proliferation of cells was measured using MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay) according to manufacturer's instructions. Briefly, keratocytes at a density of 2×10^3 /well were seeded in a 96-well plate overnight and treated with various concentrations of ACh for 24 h. To assess type of ACh receptors responsible for ACh effect on proliferation, cells were pretreated with various concentrations of atropine (muscarinic receptors antagonist) and mecamlamine (nicotinic receptors antagonist). Moreover, to assess involvement of MAP kinase cascade in ACh-induced proliferation, cells were pretreated with 25 μ M PD98059 (MAP kinase inhibitor). Next, 20 μ l of the MTS reagent was added into each well and cells were incubated for 4 h at 37 °C, 5% CO₂. To measure

the amount of formazan produced by cellular reduction of MTS, absorbance at 490 nm was measured with a micro-plate reader (BioTek, Winooski, VT, USA).

2.5. BrdU incorporation ELISA

ACh effect on cell proliferation was performed by measurement of BrdU incorporation in newly synthesized cellular DNA according to manufacturer's instructions. Briefly, 3×10^3 keratocytes were seeded into 96-well plate overnight and treated with various concentrations of ACh. Additionally, cells were pretreated with atropine, mecamlamine, or PD98059 as described in a previous section. One hour after treatments, cells were labeled with 10 μ M BrdU and incubated for 24 h at 37 °C, 5% CO₂. Next, cells were fixed and the DNA was denatured. Anti-BrdU-POD was added to bind BrdU incorporated in newly synthesized DNA. Immune complexes were detected by measuring the absorbance at 370 nm (with a reference wavelength at 492 nm) with a micro-plate reader (BioTek).

2.6. Western blot analysis

2.5×10^5 cells/well were plated in 6-well plates. In order to block muscarinic or nicotinic receptors, cells were treated with atropine or mecamlamine for 30 minutes. Afterwards, cells were treated with ACh. After indicated time, keratocytes were washed with PBS and freeze/thawed 3 times. Cells were further lysed in RIPA buffer supplemented with proteinase and phosphatase inhibitor cocktail (Fisher Scientific, Waltham, MA, USA). Protein concentration was assessed by Bradford assay (Bio-Rad, Hercules, CA, USA). Aliquots of the lysates (12 μ g for p-Erk1/2 (Thr202/Tyr204); 30 μ g for PCNA and Ki67 studies) were separated on either 4–20% or 7.5% (Ki67) SDS–polyacrylamide gel and transferred to PVDF membranes. After blocking with either 5% non-fat dry milk or 5% BSA, membranes were incubated with primary antibodies at 4 °C overnight. Then, the membranes were incubated with HRP-conjugated antibodies for 1 h at room temperature. Images were taken by Odyssey® Fc imaging system (LI-COR, Lincoln, NE, USA). Densitometry was performed using Image J analysis software (NIH).

2.7. Immunocytochemistry

10^4 cells per well were seeded in an 8-well chamber slides (Corning, Corning, NY) overnight. Cells were treated with 10^{-7} M ACh. After 24 h, medium was removed and cells were washed twice with PBS. Keratocytes were fixed with 3.7% paraformaldehyde in $1 \times$ PBS for 5 minutes at room temperature, and then blocked for 15 minutes with rabbit normal serum in PBS (1:20). Cells were incubated with Ki-67 antibody (1:200) for 1 h at 37 °C and blocked again for 15 minutes with rabbit normal serum in PBS (1:10). After washing, secondary antibody labeled with TRITC (Dako, Glostrup, Denmark) was incubated with the cells for 30 minutes at 37 °C. Finally, cells were mounted in ProLong® Diamond Antifade Mountant with DAPI (Life Technologies). A control well was also prepared for secondary antibody by replacing the primary antibody with PBS. A Zeiss Axioskop 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera were used for analysis.

2.8. Statistical analysis

All experiments were performed in triplicates. Data are presented as mean \pm SD. Statistical analysis was carried out using one-way or two-way ANOVA and Bonferroni post hoc test. Differences were considered statistically significant at a p value of < 0.05 . The experiments were repeated successfully at least three times.

3. Results

3.1. Acetylcholine enhances keratocyte proliferation

The potential effect of ACh on keratocyte proliferation was evaluated by MTS assay (Fig. 1A) and BrdU ELISA (Fig. 1B). The results showed that the lower concentrations of ACh used (10^{-8} and 10^{-7}) enhanced cell proliferation. The higher concentration of ACh (10^{-6} M), however, did not show a significant effect on proliferation in either method.

Since migration of keratocytes is another important step of corneal wound healing, the involvement of ACh in this step was also studied. ACh had no stimulatory effect on keratocyte migration as assessed by migration assay (data not shown).

3.2. Acetylcholine enhances keratocyte proliferation through activation of muscarinic acetylcholine receptors

In order to study through which type of receptors ACh exerts its proliferative effect, keratocytes were treated with different concentrations of mecamylamine (nAChR antagonist) or atropine (mAChR antagonist) for 30 minutes. Afterwards, cells were treated with various concentrations of ACh (10^{-8} , 10^{-7} , and 10^{-6} M) for 24 h. Cell proliferation was assessed by MTS assay and BrdU ELISA. The results showed that treatment of cells with mecamylamine did not abolish the ACh proliferative effect on keratocytes (Fig. 2A). However, treatment of cells with atropine resulted in the proliferative effect of ACh being reduced (Fig. 2B), suggesting mAChR involvement in ACh signaling.

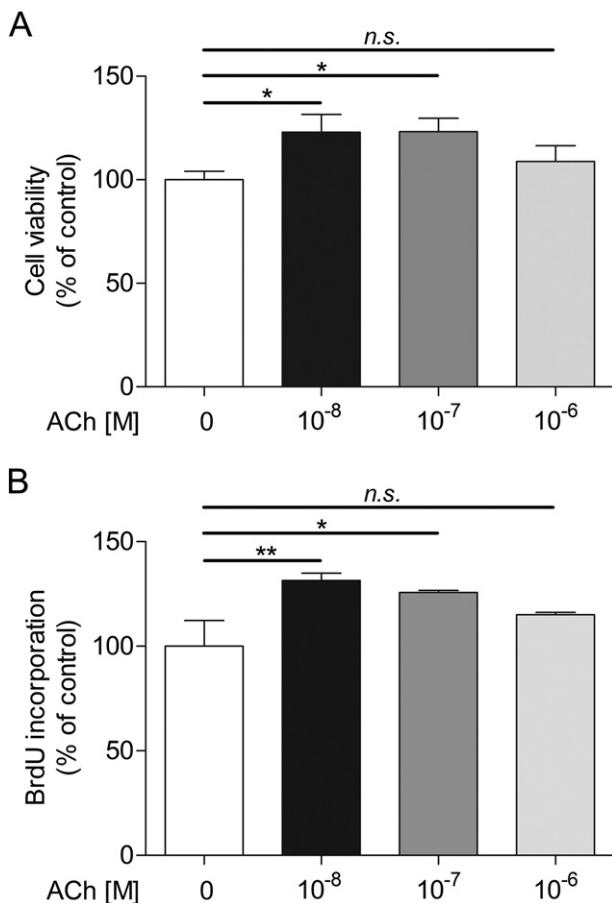


Fig. 1. Acetylcholine enhances keratocyte proliferation. Human keratocytes were cultured with different concentrations of acetylcholine (ACh) for 24 h. ACh was found to promote proliferation at the lower concentrations as assessed by MTS assay (A) and BrdU incorporation (B). Values are means \pm SD. n.s. (not significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.01$.

Moreover, to test whether removal of keratocytes from collagen matrix in the cornea has an effect on mAChR and nAChR expression in cultured keratocytes, cells were grown on either collagen I coated 6-well plates or standard polystyrene 6-well plates. Expression was assessed by RT-qPCR and western blot. No differences in mAChR and nAChR mRNA levels were found. However, western blot analysis revealed that mAChR subtype M_1 , mAChR subtype M_3 , and mAChR subtype M_5 were expressed in higher amounts by keratocytes grown on collagen I layer than by keratocytes on standard plates (data not shown). Treatment of keratocytes with 10^{-6} M ACh resulted in downregulation of mAChR subtype M_3 on protein level, but the expression of the other receptor subtypes was not affected (data not shown). The latter experiment was performed to determine whether a change in receptor expression might explain the absent proliferative effect of the higher ACh concentration.

3.3. Acetylcholine stimulation upregulates expression of PCNA and Ki-67 proliferation markers

PCNA and Ki-67 are well-known and approved cell proliferation markers [19,20]. In order to establish if ACh stimulation leads to increased expression of these markers, western blot (Fig. 3A and B) and immunocytochemistry (Fig. 3C) were performed. Western blot analysis showed that cells treated with 10^{-6} M ACh have upregulated PCNA expression. However, such effect could not be observed with 10^{-8} M ACh. Ki-67 expression was upregulated for both 10^{-6} and 10^{-8} M ACh treatment. Blocking mAChRs with 10^{-5} M atropine resulted in downregulation of both PCNA and Ki-67 proteins in cells treated with 10^{-6} M ACh, however, not in the cells treated with 10^{-8} M ACh. nAChR blockage did not affect PCNA or Ki-67 upregulation by ACh. To further analyze expression of Ki-67, ACh (10^{-7} M)-treated keratocytes were immunostained with Ki-67 antibody (Fig. 3C). Untreated cells showed no Ki-67 expression, on the other hand, cells treated with ACh showed Ki-67 expression, which was localized to the nuclei.

3.4. Acetylcholine stimulation leads to Erk1/2 phosphorylation

ACh signaling has been shown to activate MAP kinases (Erk1/2) in different cell types [9,11,12]. Therefore, we checked whether stimulation of keratocytes with ACh leads to phosphorylation (i.e. activation) of these specific kinases. Western blot analysis showed that ACh stimulation of keratocytes leads to enhanced Erk1/2 phosphorylation, as compared to control, with a peak at 30 minutes after stimulation. Pretreatment with 10^{-5} M of atropine to block mAChRs resulted in reduced Erk1/2 phosphorylation after ACh stimulation as compared to ACh stimulation without a blocker. Blocking nAChRs with 100 nM mecamylamine also led to reduced phosphorylation of Erk1/2 after ACh stimulation as compared to ACh stimulation without a blocker, although the phosphorylation after nAChR blockage was more prominent than for atropine-treated cells. (Fig. 4A). Furthermore, to study if activation of Erk1/2 is essential for ACh-enhanced proliferation, cells were treated with 25 μ M PD98059, an Erk1/2 inhibitor, and ACh of various concentrations (10^{-8} , 10^{-7} , 10^{-6} M). Inhibiting Erk1/2 resulted in no enhancement of cell proliferation by ACh (Fig. 4B), which suggests that activation of Erk1/2 is essential for ACh signaling leading to proliferation in keratocytes.

4. Discussion

This study shows that human keratocytes, when exposed to ACh, are stimulated to proliferate via mAChRs and to activate the MAPK/Erk pathway. Previous reports have shown positive effects of ACh on proliferation of murine embryonal carcinoma cells [9], human lung fibroblasts [21], and human tenocytes [22]. In human keratocytes, we observed that after 24 h, both the number of viable cells and number of cells in the S-phase of the cell cycle (BrdU incorporation) were

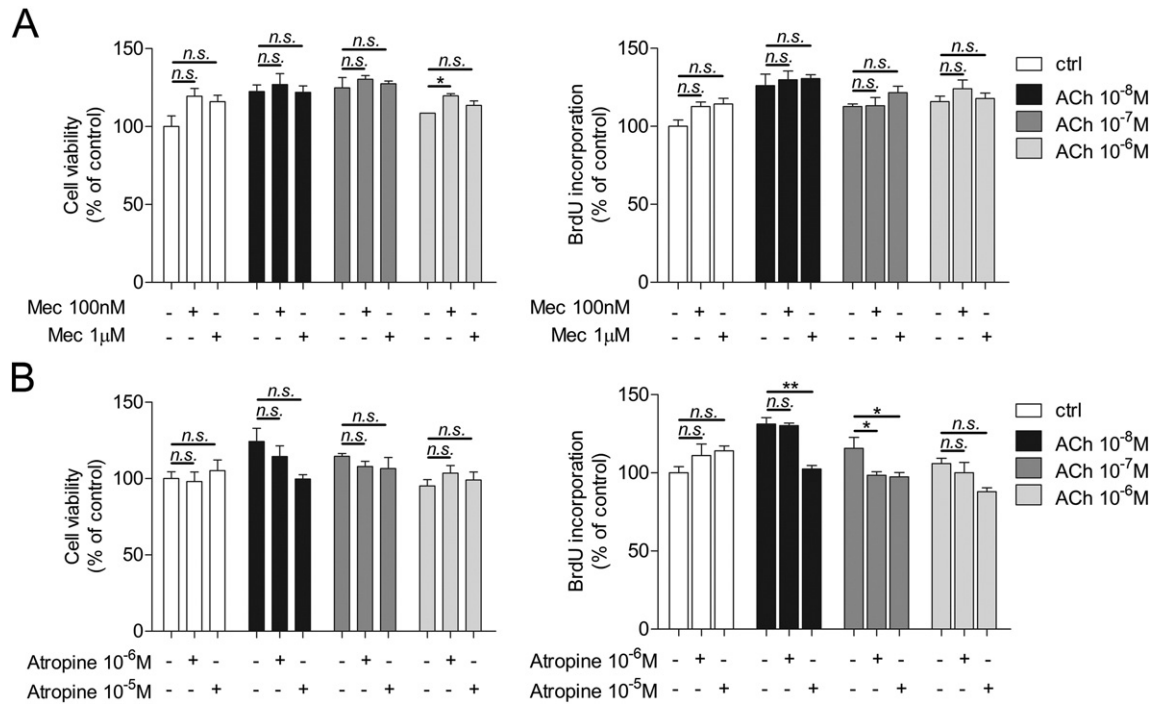


Fig. 2. Acetylcholine enhances keratocyte proliferation through activation of muscarinic acetylcholine receptors. Human keratocytes were pretreated with different concentrations of the nicotinic acetylcholine receptor antagonist mecamylamine (Mec; A) or the muscarinic acetylcholine receptor antagonist atropine (B) for 30 minutes and afterwards treated with various concentrations of acetylcholine (ACh) for 24 h. Cell proliferation was assessed by MTS and BrdU incorporation. Atropine was found to reduce ACh-stimulated proliferation, whereas mecamylamine did not have that effect, suggesting that the proliferative effect of ACh is mediated by muscarinic ACh receptors. Values are means \pm SD. *n.s.* (not significant), $p > 0.05$; * $p < 0.05$; ** $p < 0.01$.

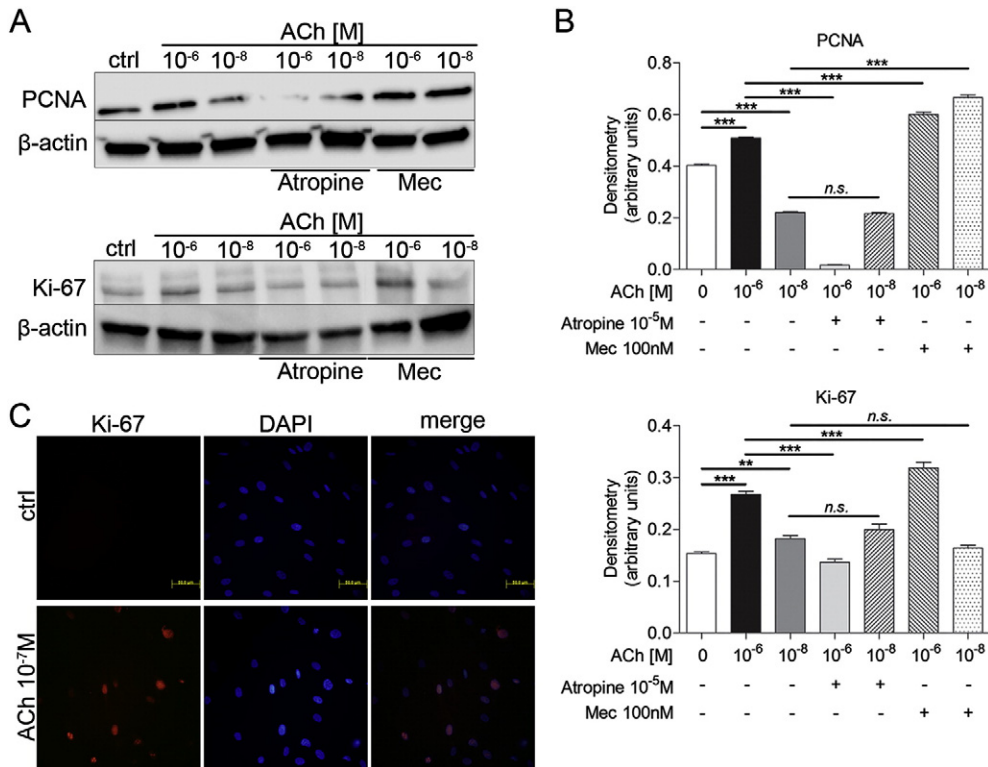


Fig. 3. Acetylcholine stimulation upregulates expression of proliferation markers PCNA and Ki-67. (A) Human keratocytes were pretreated with either atropine or mecamylamine (Mec) for 30 minutes and afterwards treated with two concentrations of acetylcholine (ACh; 10⁻⁶ and 10⁻⁸ M) for 24 h. Expression of PCNA (36 kDa) and Ki-67 (345 and 395 kDa) was determined by western blot. β -actin (42 kDa) served as a loading control. ACh increased expression of both Ki-67 and PCNA. This increase was blocked with atropine but not mecamylamine, thus indicating that the ACh effect was exerted through muscarinic ACh receptors. (B) Densitometric analysis of PCNA and Ki-67 expression on the western blot. (C) Immunocytochemistry of human keratocytes in culture shows that when treated with 10⁻⁷ M of ACh for 24 h, the cells display clear Ki-67 (red) reactions, which are not seen without the ACh treatment (left column). Representative pictures are shown. DAPI (blue) counter-staining of nuclei shown in the middle column; merge to the right. Values are means \pm SD. *n.s.* (not significant), $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$.

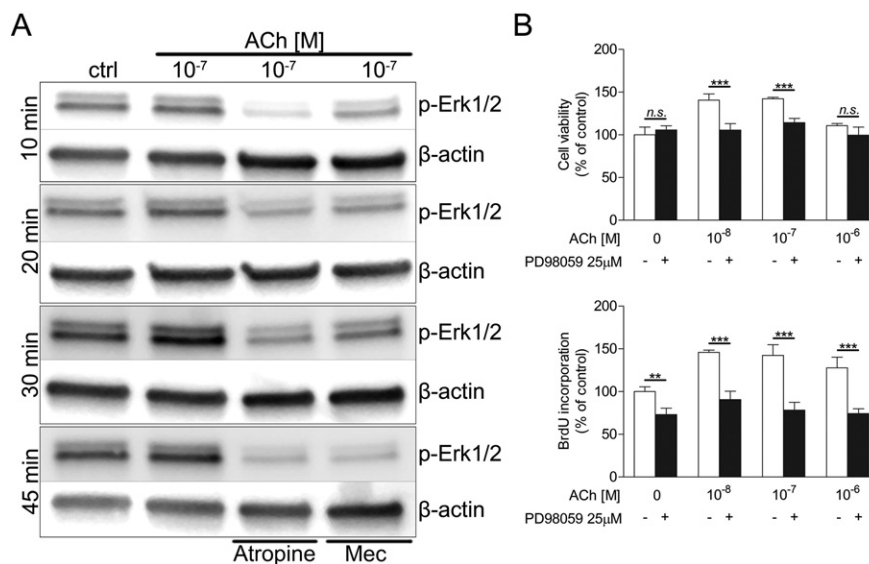


Fig. 4. Acetylcholine stimulation leads to Erk1/2 phosphorylation. (A) Human keratocytes were pretreated with 10^{-5} M atropine or 100 nM mecamlamine (Mec). Afterwards, cells were treated with 10^{-7} M of acetylcholine (ACh) for 10, 20, 30, and 45 minutes. Phosphorylation status of Erk1/2 (42 and 44 kDa) was determined by western blot. β -actin (42 kDa) served as a loading control. ACh increased phosphorylation of Erk1/2 with a peak at 30 min. Cells treated with atropine or mecamlamine and ACh showed decreased Erk1/2 phosphorylation as compared to cells only treated with ACh. However, atropine treatment resulted in less Erk1/2 phosphorylation when compared to mecamlamine treatment. (B) Activation of Erk1/2 was found to be essential for ACh-induced proliferation, as seen when keratocytes were pretreated with PD98059, an Erk1/2 inhibitor, for 1 h and afterwards treated with various concentrations of ACh (10^{-8} , 10^{-7} , and 10^{-6} M) for 24 h. Cell proliferation was measured by MTS assay and BrdU incorporation, and the ACh stimulated proliferation was found to be significantly decreased when preincubated with the Erk1/2 blocker. Values are means \pm SD. n.s. (not significant), $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$.

significantly higher in the cells exposed to ACh at concentrations 10^{-8} M and 10^{-7} M as compared with unstimulated cells. Interestingly, higher concentration of ACh (10^{-6} M) did not stimulate keratocyte proliferation. Proliferating cell nuclear antigen (PCNA), which is a well-accepted marker of proliferation and is a member of the DNA sliding clamp family of proteins that assist in DNA replication [19], was upregulated after ACh stimulation of keratocytes. Similarly, Ki-67 which is also a marker of proliferation and is present during all active phases of the cell cycle (G_1 , S, G_2 , and mitosis) but is absent from resting cells (G_0) [20], was upregulated in response to ACh. However, the higher concentration of ACh (10^{-6} M) resulted in higher PCNA and Ki-67 expression than treatment with lower concentration of ACh (10^{-8} M). The lower efficiency of high ACh concentration to increase the number of viable cells and BrdU incorporation into newly synthesized DNA might occur due to ACh receptor desensitization. It is known that high concentrations of ACh cause downregulation of muscarinic receptors which is generally accompanied by decreased sensitivity to muscarinic agonists [23–25]. Indeed, we did observe that a high concentration of ACh downregulates the expression of mAChR subtype M_3 , a subtype of mAChR that has in fact been reported to mediate cell proliferation [26]. Alternatively, these effects might be due to activation of a different mechanism by ACh stimulation, since low ACh concentrations have been shown to cause a calcium-independent stimulation of proliferation of rat lymphoblasts, whereas higher ACh concentrations act through calcium-dependent mechanisms [27]. However, our experiments showed that high concentration of ACh resulted in higher PCNA and Ki-67 protein expression than low concentration treatment. As the endpoint of proliferation is an increase in cell number, and both PCNA and Ki-67 are taking part in committing the cell into proliferation, perhaps a high concentration of ACh results in post-translational modifications of these two proteins which would stop the cell from dividing [28,29]. Additionally, the removal of keratocytes from the cornea and their processing during culturing causes downregulation of mAChRs, which might explain why we do not observe a more robust proliferation of cultured keratocytes after ACh stimulation.

The role of mAChRs in control of the cell proliferation is well known. For example, activation of mAChR subtype M_2 and mAChR subtype M_3 has been shown to increase proliferation of tumor cells [30] and

fibroblasts [21]. However, the involvement of nAChR in control of cell proliferation is less well understood. Nevertheless, there are data suggesting that activation of these receptors might lead to migration, survival, and differentiation in oligodendrocyte precursors [31] as well as proliferation in human non-small cell lung cancer cells [32]. Our results indicate that ACh-enhanced proliferation of keratocytes is driven through activation of mAChRs rather than nAChRs. We observed that mecamlamine, a nonselective and noncompetitive antagonist of nAChRs, did not abolish the proliferative effects of ACh, as the number of viable cells, the numbers of cells with incorporated BrdU, and the expression of PCNA and Ki-67 remained either unchanged (when low concentrations of ACh were used) or showed slightly higher cell numbers and protein expression (at higher concentrations of ACh). However, when atropine, a competitive mAChR antagonist, was used, ACh treatment did not stimulate keratocytes to proliferate; neither viable cell count nor BrdU incorporation increased, and expression of both PCNA and Ki-67 was downregulated. It is known that ACh-induced proliferation is mediated by activation of MAPK-dependent pathways [11,33] and that activation of MAPK agonists mediate cell proliferation [34, 35]. Furthermore, pathways involving cholinergic receptors seem to be dependent on the cell growth [9,36]. MEK1 and MEK2, also called MAPK or Erk kinases, are dual-specificity protein kinases that function in a mitogen-activated protein kinase cascade controlling cell growth and differentiation [37]. Our results showed that in keratocytes, ACh signals through activation of MAPK (Erk1/2) and that this activation is mediated mostly via mAChRs and to lesser extent via nAChRs. Moreover, activation of this pathway was found to be essential for ACh-stimulated proliferation, as the proliferative effect of ACh on keratocytes was abolished when PD98059, a highly selective inhibitor of MEK1 activation and the MAP kinase cascade, was used.

The cornea which is normally well protected from injury might under some circumstances, such as infection or trauma, become injured. When this happens, a healing cascade is activated. Briefly, this complex process starts with apoptosis of the keratocytes adjacent to the injury site. Subsequently, the remaining keratocytes start to proliferate and migrate in order to fill the wound. Eventually, new extracellular matrix is produced and the functionality of the cornea is restored [38,39]. However, the wound-healing process can malfunction and, as a result,

corneal scarring may occur, which in turn might cause visual impairment or even blindness [40]. It is therefore of great value to study substances that are involved in, or may contribute to, effective corneal wound healing. In this regard, ACh is of interest since it has been implicated in corneal epithelial and endothelial wound healing [17,18]. However, the mechanisms of ACh activity remain unclear and the role of ACh in the stroma is also unknown. Therefore, our findings that ACh enhances proliferation of corneal stromal keratocytes *in vitro*, through activation of mAChRs and the MAPK/Erk signaling pathway, provide new insights into understanding the mechanisms of ACh signaling in keratocytes and its possible implication in promoting corneal stromal wound healing.

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