

Activation of the EGF Receptor by Histamine Receptor Subtypes Stimulates Mucin Secretion in Conjunctival Goblet Cells

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Submitted: May 9, 2018

Accepted: June 18, 2018

Citation: He M, Lippestad M, Li D, Hodges RR, Utheim TP, Dartt DA. Activation of the EGF receptor by histamine receptor subtypes stimulates mucin secretion in conjunctival goblet cells. *Invest Ophthalmol Vis Sci.* 2018;59:3543–3553. <https://doi.org/10.1167/iovs.18-2476>

PURPOSE. The purpose of this study was to determine if histamine receptors interact with the epidermal growth factor receptor (EGFR) in cultured rat conjunctival goblet cells.

METHODS. Goblet cells from rat conjunctiva were grown in organ culture. First-passage goblet cells were used in all experiments. Phosphorylated (active) and total EGFR, AKT, and extracellular signal-regulated kinase (ERK)1/2 were measured by Western blot analysis. Cells were preincubated with the EGFR antagonist AG1478 for 30 minutes or small interfering RNA specific to the EGFR for 3 days prior to stimulation with histamine or agonists specific for histamine receptor subtypes for 2 hours. Goblet cell secretion was measured using an enzyme-linked lectin assay. Goblet cells were incubated for 1 hour with the calcium indicator molecule fura-2/AM, and intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) was determined. Data were collected in real time and presented as the actual $[Ca^{2+}]_i$ with time and as the change in peak $[Ca^{2+}]_i$.

RESULTS. Histamine increased the phosphorylation of the EGFR. Mucin secretion and increase in $[Ca^{2+}]_i$ stimulated by histamine, and agonists specific for each histamine receptor subtype were blocked by inhibition of the EGFR. Increase in $[Ca^{2+}]_i$ stimulated by histamine and specific agonists for each histamine receptor was also inhibited by TAPI-1, a matrix metalloproteinase (MMP) inhibitor. The histamine-stimulated increase in activation of AKT, but not ERK1/2, was blocked by AG1478.

CONCLUSIONS. In conjunctival goblet cells, histamine, using all four receptor subtypes, transactivates the EGFR via an MMP. This in turn phosphorylates AKT to increase $[Ca^{2+}]_i$ and stimulate mucin secretion.

Keywords: mucin secretion, allergy, goblet cells

The ocular surface consists of the cornea and conjunctiva. These tissues are continuously exposed to the external environment and therefore must constantly react to environmental factors, allergens, bacteria, viruses, and other pathogens. The conjunctiva, which contains stratified squamous and goblet cells, can respond robustly to these challenges. One mechanism by which both types of conjunctival cells protect the ocular surface is the secretion of mucin. Stratified squamous epithelia shed the membrane-spanning mucins MUC1, MUC4, MUC16, and MUC20.¹ Goblet cells respond to the external environment by secreting the large secretory mucin MUC5AC.² The membrane-spanning mucins are either part of the glycocalyx or are shed into the tear film, whereas the secretory mucin is secreted into the mucin layer of tear film. Mucins form a physical and chemical barrier to trap allergens and pathogens before they reach the ocular surface.

In spite of the protective measures of mucins, allergens or other proinflammatory compounds reach the ocular surface, inducing allergic conjunctivitis, a common eye disease that

affects at least 10% to 20% of people globally.³ One type of allergic conjunctivitis is seasonal allergic conjunctivitis (SAC), which is predominantly an IgE-mediated hypersensitivity reaction.⁴ After the allergen cross-links to the specific IgE, mast cells degranulate and release inflammatory mediators such as histamine, prostaglandins, and leukotrienes.⁴ These mediators lead to the symptoms of itching, redness, lid swelling, chemosis, and tearing.

Conjunctival goblet cells express receptors for histamine, prostaglandins, and leukotrienes.^{5,6} Of the four types of histamine (H) receptors (R), H1R, H2R, H3R, and H4R, all are expressed on goblet cells. Activation of each subtype, using receptor specific agonists, leads to an increase in mucin secretion.⁵ Activation of histamine receptors by histamine increases intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$),⁷ and chelating this increase in $[Ca^{2+}]_i$ blocks mucin secretion.⁸ In addition, activation of the H1R by a specific H1 agonist is counter-regulated by the pro-resolution mediators resolvin RvD1 and lipoxin A₄.^{9,10} This counter-regulation occurs via phosphoryla-

tion of the receptor by β -adrenergic receptor kinase and protein kinase C, which blocks the increase in $[Ca^{2+}]_i$ and mucin secretion.^{9,10}

EGF and its family of ligands—heparin-binding EGF (HB-EGF), amphiregulin, and TGF- α —bind to four receptor tyrosine kinase family members termed ErbB receptors.¹¹ This family consists of the epidermal growth factor receptor (EGFR, ErbB1, HER1), ErbB2 (HER2/neu), ErbB3, and ErbB4.¹¹ The ligands are synthesized as glycosylated membrane-bound precursors that are cleaved to release a soluble proform. The proform is further cleaved to form a biologically active form.¹¹ The process by which ligands are cleaved is known as ectodomain shedding and is mediated by matrix metalloproteinases (MMP).¹¹ It is well established that many G-protein-coupled receptors transactivate the EGFR receptor. They do so through activation of the MMP, an inhibitor of disintegrin and metalloproteinase 17 (ADAM17).^{12,13} After ligand binding, ErbB receptors form homo- and heterodimers, causing autophosphorylation, recruiting adaptor proteins, and activating a large number of signaling pathways including the phosphoinositide-3 kinase (PI3K) pathway.¹¹ Stimulation of the PI3K pathway leads to phosphorylation of AKT, also known as protein kinase B, and extracellular signal-regulated kinase (ERK)1/2. In the conjunctiva, goblet cells express all four ErbB receptors, and activation leads to cell proliferation, increase in $[Ca^{2+}]_i$, and mucin secretion.^{14–16} In addition, in conjunctival goblet cells, EGFR activates PI3K and AKT to stimulate goblet cell proliferation.^{14,17}

In the human airway, EGFR expression is increased in asthma and correlates with severity of disease.¹⁸ The EGFR induces airway remodeling through the release of EGF receptor ligands.¹⁹ In addition, in a small study, the EGFR, not the H1 or H2 histamine receptors, were upregulated in seasonal allergic rhinitis.²⁰ In conjunctiva from patients with vernal keratoconjunctivitis, strong membranous and cytoplasmic EGFR expression was observed in the epithelium.²¹

Since conjunctival goblet cells express both histamine and ErbB receptors and activation of both types of receptors stimulates mucin secretion, we determined if the two types of receptors interact to cause an increase in $[Ca^{2+}]_i$ and mucin secretion.

MATERIALS AND METHODS

Materials

Histamine and the specific histamine receptor agonists 2-([3-trifluoromethyl]-phenyl histamine dimaleate (H1R agonist), amthamine dihydrobromide (H2R agonist), and (R)- α -methyl-histamine dihydrochloride (H3R agonist) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). The receptor agonist 4-methylhistamine dihydrochloride (H4) and TAPI-1 (ADAM17 inhibitor) were from Tocris Bioscience (Ellisville, MO, USA). EGF was from Sigma-Aldrich, and Amplex Red and fura-2-acetoxymethyl ester (fura-2/AM) were purchased from Invitrogen (Grand Island, NY, USA). An EGFR antagonist was from LC Laboratories (Tyrphostin AG 1478; Woburn, MA, USA). Antibodies directed against the EGFR, phosphorylated EGFR (Tyr1068), AKT, phosphorylated AKT, and Western blotting application solution kits were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against ERK and phosphorylated ERK and small interfering RNA (siRNA) transfection medium were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection reagent, rat EGFR siRNA-SMART pool, and nontargeting pool were from Dharmacon RNAi Technology (Dharma-

TECT1, ON-TARGETplus, ON-TARGETplus, respectively; Lafayette, CO, USA).

Animals

Male Sprague-Dawley rats weighing between 125 and 150 g were obtained from Taconic Farms (Germantown, NY, USA). Rats were anesthetized with CO₂ for 1 minute, decapitated, and the bulbar and fornical conjunctiva removed from both eyes. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

Cell Culture

Goblet cells from excised rat conjunctiva were grown in organ culture as described previously.^{8,10,22–24} Pieces of minced conjunctival tissue were placed in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μ g/mL penicillin-streptomycin. The tissue plug was removed after nodules of cells were observed. First-passage goblet cells were used in all experiments. The identity of cultured cells was periodically confirmed by evaluating staining with antibody to cytokeratin 7 (which detects goblet cell bodies) and the lectin *Ulex europaeus* agglutinin (UEA)-1 (which detects goblet cell secretory products) to ensure that goblet cells predominated.

EGFR, AKT, and ERK1/2 Activation by Western Blot Analysis

The expression of phosphorylated (active) EGFR, AKT, and ERK1/2 and total EGFR, AKT, and ERK1/2 was measured by Western blot analysis. Primary cultures of rat conjunctival goblet cells were trypsinized and seeded into six-well plates. Cells were grown to approximately 80% confluency and were serum starved for 24 hours. For activation of the EGFR, cells were then stimulated with EGF (10^{-7} M) for 5 minutes and histamine (10^{-6} M) for 1 to 60 minutes. For activation of AKT and ERK1/2, cells were preincubated with the EGFR antagonist AG1478 (10^{-8} and 10^{-7} M) for 30 minutes prior to addition of histamine (10^{-6} M) for 5 minutes. The reaction was stopped by the addition of ice-cold PBS (145 mM NaCl, 7.3 mM Na₂PO₄ at pH 7.2). Cells were homogenized in cell lysis buffer (Cell Signaling Technology), and cells were scraped. The homogenates were collected, sonicated, and centrifuged at 14,500g for 10 minutes at 4°C. Proteins were separated by SDS-PAGE using a 10% gel and processed for Western blotting as described previously.^{15,25,26} Primary antibodies for EGFR or phosphorylated EGF receptor were diluted to 1:200. Antibodies against phosphorylated AKT and ERK1/2 were used at 1:500 dilution. β -Actin, total AKT, and ERK1/2 antibodies were used at a dilution of 1:1000. Secondary antibody was used at a dilution of 1:2000. Immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with ImageJ software (<http://rsbweb.nih.gov/ij/>); provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Measurement of High-Molecular-Weight Glycoconjugate Mucin Secretion

Cultured goblet cells were seeded in 24-well plates and grown to 90% confluence. Cells were serum starved in serum-free RPMI 1640 supplemented with 0.35% bovine serum albumin (BSA) for 2 hours before use. Cells were preincubated with

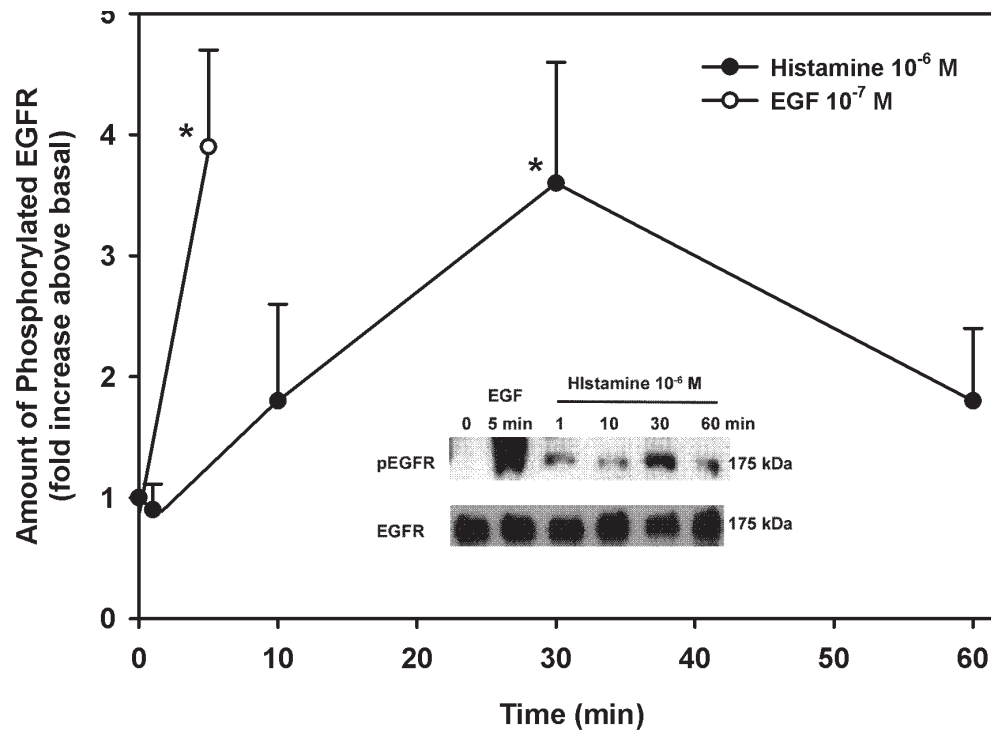


FIGURE 1. Histamine increases phosphorylation of the EGFR. Cultured rat conjunctival goblet cells were stimulated with either histamine 10^{-6} M for 0 to 60 minutes or EGF 10^{-7} M for 5 minutes. Cells were homogenized and proteins analyzed by Western blot using antibodies against phosphorylated (active) EGFR and total EGFR. Data are mean \pm SEM from six independent experiments. Asterisk indicates significant difference from $t = 0$. Inset is a representative blot.

EGFR antagonist AG1478 for 30 minutes prior to stimulation with histamine or the histamine receptor agonists for 2 hours. Goblet cell secretion was measured using an enzyme-linked lectin assay (ELLA) with the lectin UEA-1 as described previously.^{8,10,22,23} UEA-1 detects high-molecular-weight glycoconjugates, including mucins produced by rat goblet cells. The standards and supernatants were spotted on microplates (Nunc; Thermo Scientific, Waltham, MA, USA) and dried overnight at 60°C . The ELLA was performed using UEA-1 conjugated to horseradish peroxidase. UEA-1 was detected using Amplex Red, which when oxidized by peroxidase in the presence of hydrogen peroxide produces a highly fluorescent molecule. The fluorescence was quantified on a fluorescent ELISA reader (Synergy MX; Bio-Tek, Winooski, VT, USA) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The cells were scraped, sonicated, and the cell homogenate analyzed for the total amount of protein using the Bradford protein assay. High-molecular-weight glycoconjugate mucin secretion was normalized to total protein in the homogenate. Bovine submaxillary mucin was used for the standard curve. High-molecular-weight glycoconjugate mucin secretion, which will be referred to as mucin secretion, was expressed as fold increase over basal value, which was set to 1.

siRNA Experiments for Depletion of EGFR

First-passage goblet cells were grown in 24-well plates to 60% confluence. siRNA specific to the EGFR or negative control siRNA, was added at a final concentration of 100 nM in antibiotic-free RPMI 1640 as described previously.^{7,8} Media was removed after 18 hours and replaced with fresh, complete RPMI 1640 and incubated for 48 hours before use. To ensure

the successful depletion of EGFR from the goblet cells, one well per condition was scraped, and Western blotting analysis using antibody against EGFR was performed as described above.

Measurement of $[\text{Ca}^{2+}]_i$

Goblet cells were incubated for 1 hour at 37°C with Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES) (119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , 10 mM HEPES, and 5.5 mM glucose [pH 7.45]) plus 0.5% BSA containing 0.5 μM fura-2/AM, 8 μM pluronic acid F127, and 250 μM sulfinpyrazone, as described previously.^{8-10,22,23} Cells were washed with KRB-HEPES containing sulfinpyrazone. Calcium measurements were made with a ratio imaging system (In Cyt Im2; Intracellular Imaging, Cincinnati, OH, USA) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. At least 10 cells were used for each condition. Inhibitors, dissolved in KRB-HEPES buffer, were added 30 minutes before the agonists. Data were collected in real time and presented as the actual $[\text{Ca}^{2+}]_i$ with time and as the change in peak $[\text{Ca}^{2+}]_i$. Change in peak $[\text{Ca}^{2+}]_i$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[\text{Ca}^{2+}]_i$. Although data are not shown, the plateau $[\text{Ca}^{2+}]_i$ was affected similarly to the peak $[\text{Ca}^{2+}]_i$.

Statistical Analysis

Results are presented as mean \pm SEM. Data were analyzed by Student's t -test. $P < 0.05$ was considered statistically significant.

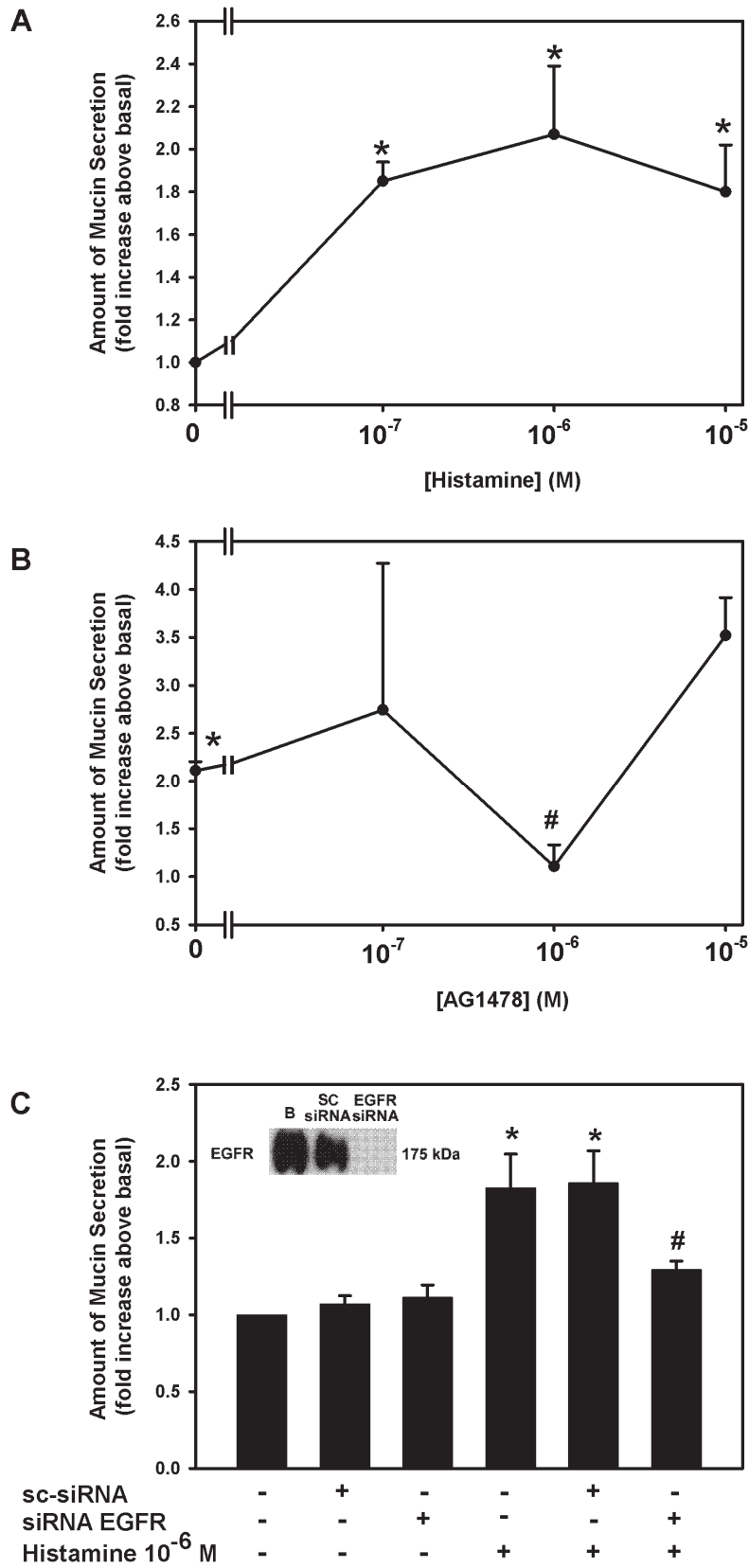


FIGURE 2. Histamine-stimulated mucin secretion is blocked by inhibition of the EGFR. Cultured rat conjunctival goblet cells were stimulated with increasing concentrations of histamine (10^{-7} to 10^{-5} M) for 2 hours, and mucin secretion was measured (A). Cells were preincubated with AG1478 (10^{-7} to 10^{-5} M) for 30 minutes prior to stimulation with histamine (10^{-6} M), and mucin secretion was measured (B). Cells were treated with 100 nM scrambled (sc) or EGFR siRNA and stimulated with histamine (10^{-6} M) for 2 hours, and mucin secretion was measured (C). *Inset* in C is representative Western blot analysis of siRNA experiments using an antibody against EGFR. Data are mean \pm SEM from three (A, B) or six (C) independent experiments. *Asterisk* indicates significant difference from basal value. *Pound sign* indicates significant difference from histamine alone.

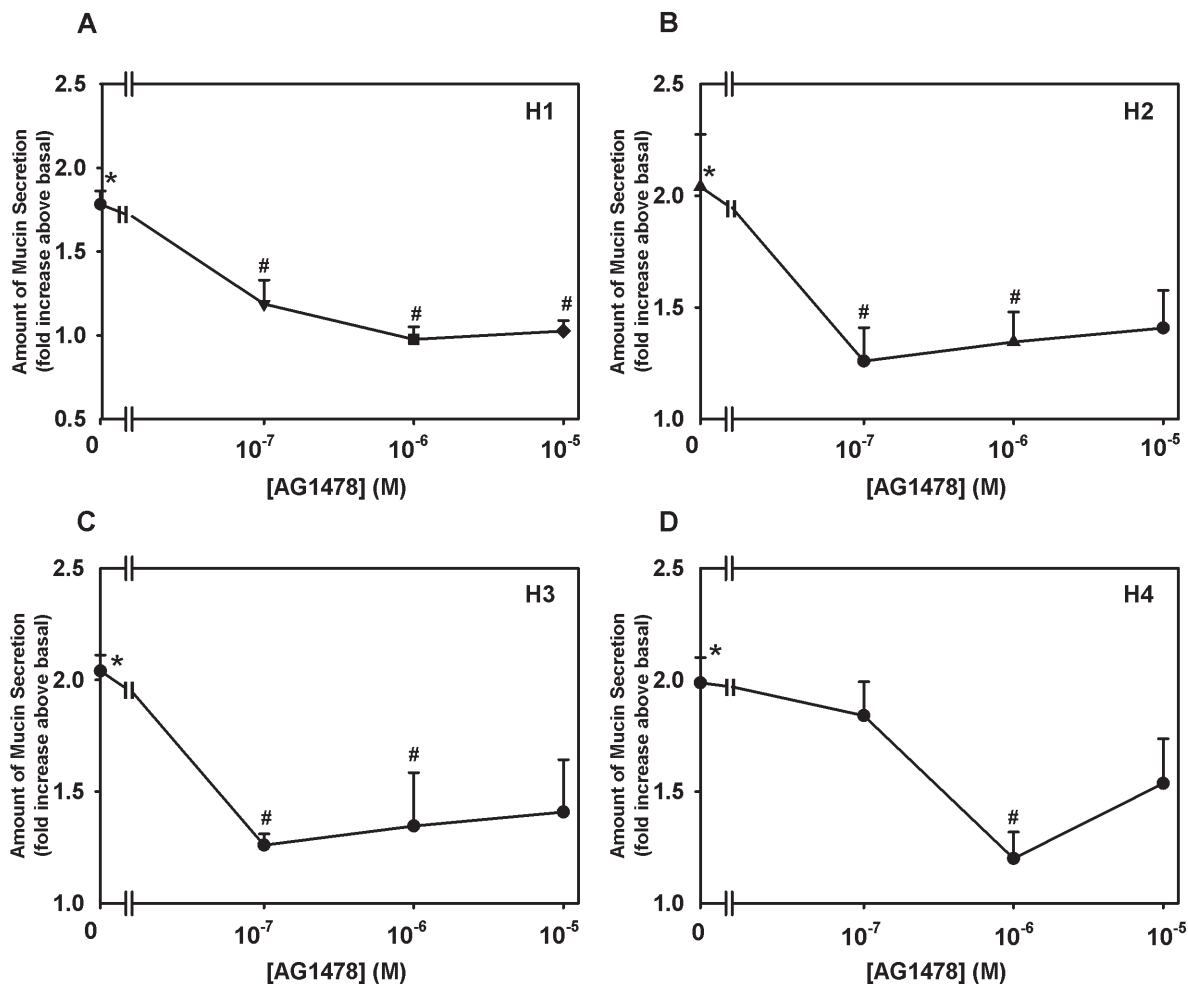


FIGURE 3. Histamine receptor subtype-stimulated secretion is blocked by inhibition of the EGFR. Cultured rat goblet cells were preincubated with AG1478 (10^{-7} to 10^{-5} M) for 30 minutes prior to stimulation with agonists specific to each receptor subtype for 2 hours, and mucin secretion was measured. The H1 receptor agonist histamine dimaleate (10^{-6} M) is shown in **A**. The H2 receptor agonist, amthamine (10^{-4} M) is shown in **B**. The H3 receptor agonist, α -methylhistamine (10^{-6} M), is shown in **C**, while the H4 receptor agonist, 4-methylhistamine (10^{-5} M) is shown in **D**. Data are mean \pm SEM from five (**A**, **B**) or three (**C**, **D**) independent experiments. Asterisk indicates significant difference from basal value. Pound sign indicates significant difference from agonist alone.

RESULTS

Histamine Induces EGFR Activation in Conjunctival Goblet Cells

To determine if histamine activates the EGFR in conjunctival goblet cells, cells were incubated with histamine (10^{-6} M) for 0 to 60 minutes or, as a positive control, EGF (10^{-7} M) for 5 minutes. Cells were homogenized, and Western blot analysis was performed using a specific antibody against phosphorylated EGFR. Histamine increased the phosphorylation of the EGFR in a time-dependent manner 1.8 ± 0.8 , 3.6 ± 1.0 , 1.8 ± 0.6 -fold above basal value at 10, 30, and 60 minutes after addition of histamine, respectively (Fig. 1). At the 5-minute time point, EGF significantly increased the phosphorylation of the EGFR 3.9 ± 0.8 -fold above basal value (Fig. 1). Thus histamine appears to transactivate the EGFR in cultured conjunctival goblet cells.

Inhibition of the EGFR Blocks Histamine-Stimulated Mucin Secretion From Conjunctival Goblet Cells

We previously showed that histamine stimulated mucin secretion.⁶ In the current study, histamine (10^{-7} to 10^{-5} M)

significantly stimulated mucin secretion with a maximum of 2.1 ± 0.3 -fold above basal value at 10^{-6} M (Fig. 2A). Histamine at 10^{-6} M was used in subsequent experiments. As histamine activated the EGFR, the effects of inhibition of EGFR on histamine-stimulated mucin secretion were explored. Goblet cells were preincubated with the EGFR inhibitor AG1478 (10^{-7} to 10^{-5} M) for 30 minutes prior to addition of histamine for 2 hours. Histamine significantly increased mucin secretion 2.1 ± 0.1 -fold above basal value. AG1478 (10^{-6} M) significantly inhibited this secretion (Fig. 2B).

To confirm the data with AG1478 that indicates that the EGFR plays a role in histamine-stimulated mucin secretion, goblet cells were incubated with EGFR siRNA. EGFR was successfully knocked down using 100 nM EGFR siRNA while scrambled siRNA had no effect on the expression of the EGFR (Fig. 2C inset). Neither scrambled nor EGFR siRNA alone had any effect on basal mucin secretion (Fig. 2C). In non-transfected cells, histamine significantly increased mucin secretion at 1.8 ± 0.2 -fold increase above basal. Incubation with scrambled siRNA did not alter goblet cell secretion stimulated by histamine (Fig. 2C). In contrast, EGFR siRNA significantly blocked histamine-stimulated secretion to 1.3 ± 0.1 -fold increase above basal (Fig. 2C). These data imply that

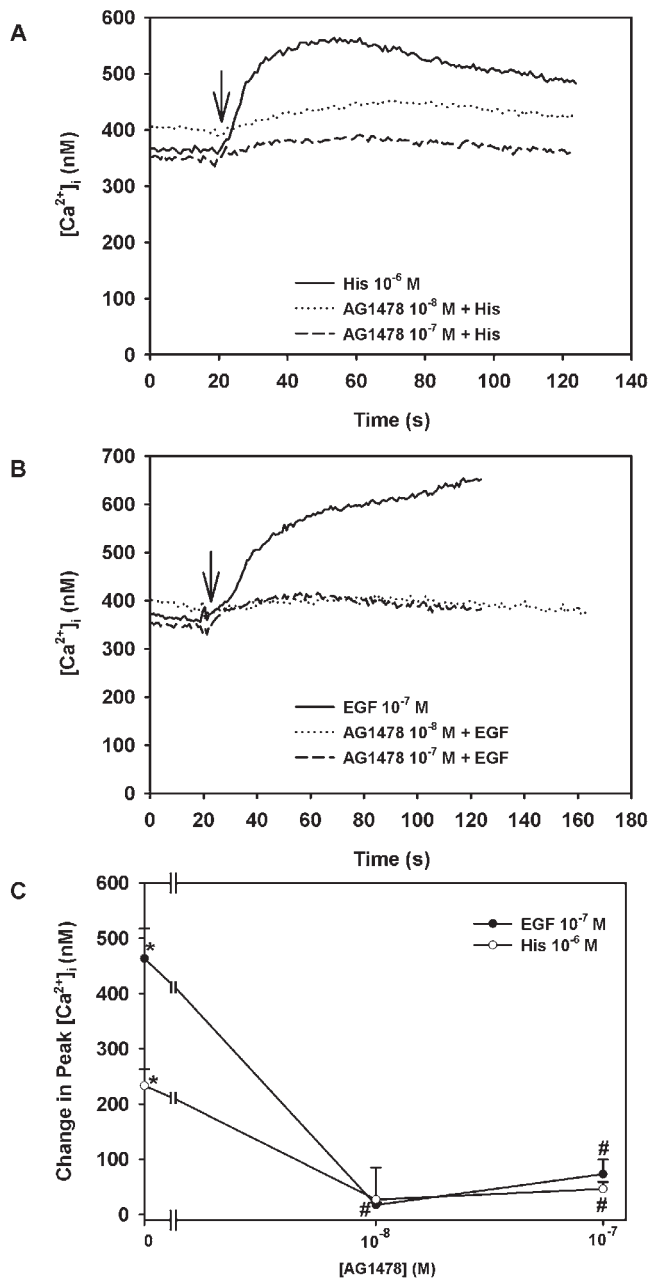


FIGURE 4. Histamine-stimulated increase in $[Ca^{2+}]_i$ is blocked by inhibition of the EGFR. Cultured rat conjunctival goblet cells were preincubated with AG1478 (10^{-8} and 10^{-7} M) for 30 minutes prior to stimulation with histamine (10^{-6} M) (A) or the positive control EGF (10^{-7} M) (B). $[Ca^{2+}]_i$ over time is shown in A and B. Change in peak $[Ca^{2+}]_i$ was determined and is shown in C. Data are mean \pm SEM from three independent experiments. Asterisk indicates significant difference from basal value. Pound sign indicates significant difference from agonist alone. Arrow indicates addition of either histamine or EGF.

histamine transactivates the EGFR to increase mucin secretion.

Inhibition of the EGFR Inhibits Mucin Secretion Stimulated by Specific Agonists for Each Histamine Receptor Subtype in Conjunctival Goblet Cells

Previous studies demonstrated that all four histamine receptor subtypes are present in conjunctival goblet cells

and activation of each subtype with specific agonists stimulated mucin secretion.⁵ To determine if one or more histamine receptor subtypes interacts with the EGFR, goblet cells were preincubated with AG1478 (10^{-8} to 10^{-6} M) and stimulated with agonists specific to each receptor subtype at the concentration previously demonstrated to stimulate maximum secretion.⁵ The H1 receptor agonist, histamine dimaleate (10^{-6} M), significantly stimulated mucin secretion 1.8 ± 0.1 -fold above basal. All three concentrations of AG1478 significantly blocked the effect of histamine dimaleate (Fig. 3A). The H2 receptor agonist amthamine (10^{-4} M) significantly stimulated goblet cell secretion by 2.0 ± 0.2 -fold above basal value. AG1478 significantly blocked the effect of H2 amthamine at all concentrations (Fig. 3B). The H3 receptor agonist α -methylhistamine (10^{-6} M) increased mucin secretion 2.0 ± 0.1 -fold above basal value (Fig. 3C). AG1478 significantly inhibited α -methylhistamine-stimulated mucin secretion at 10^{-7} and 10^{-6} M (Fig. 3C). The H4 receptor agonist 4-methylhistamine (10^{-5} M) increased mucin secretion 2.0 ± 0.1 -fold above basal value (Fig. 3D). AG1478 significantly blocked this increase at 10^{-6} M (Fig. 3D). In the same experiments, as a positive control histamine (10^{-6} M) increased mucin secretion by 1.8 ± 0.1 -fold above basal value and was significantly blocked by AG1478 (data not shown). Thus all four histamine receptor subtypes use the EGFR to stimulate mucin secretion.

Inhibition of the EGFR Blocks Histamine-Stimulated Increase in $[Ca^{2+}]_i$ in Conjunctival Goblet Cells

Histamine is known to increase $[Ca^{2+}]_i$, and inhibition of $[Ca^{2+}]_i$ blocks mucin secretion in conjunctival goblet cells.^{7,8} To determine if histamine activates the EGFR to stimulate the increase in $[Ca^{2+}]_i$, conjunctival goblet cells were preincubated with AG1478 for 30 minutes prior to addition of histamine or, as a positive control, EGF. $[Ca^{2+}]_i$ over time after addition of histamine or EGF is shown in Figures 4A and B, respectively. Histamine increased $[Ca^{2+}]_i$ by 233.2 ± 30.0 nM. AG1478 10^{-7} M significantly decreased histamine-stimulated increase in $[Ca^{2+}]_i$ to 46.2 ± 12.9 nM. In cells cultured from the same animals, EGF stimulated increase in $[Ca^{2+}]_i$ to 463.3 ± 54.3 nM (Fig. 4C). This was also significantly inhibited by AG1478 at 10^{-8} and 10^{-7} M to 17.3 ± 4.6 and 73.0 ± 26.5 nM, respectively (Fig. 4C). Therefore, the stimulation of the EGFR leads to the increase in $[Ca^{2+}]_i$ stimulated by all the histamine receptor subtypes.

Inhibition of EGFR Blocks Increase in $[Ca^{2+}]_i$ Stimulated by Specific Histamine Receptor Agonists in Conjunctival Goblet Cells

As EGF activates all histamine receptor subtypes to stimulate mucin secretion, the effect of inhibition of EGFR on specific agonist-stimulated increase in $[Ca^{2+}]_i$ was determined in cells from the same animals as used in Figure 4. The H1 receptor agonist, histamine dimaleate (10^{-6} M), significantly increased $[Ca^{2+}]_i$ to 217.5 ± 40.5 nM. This increase was completely inhibited by AG1478 at 10^{-8} and 10^{-7} M (Fig. 5A). The H2 receptor agonist, amthamine (10^{-4} M) significantly stimulated $[Ca^{2+}]_i$ to 129.3 ± 20.1 nM (Fig. 5B). AG1478 significantly blocked the increase to 9.7 ± 23.6 nm at 10^{-8} M and completely inhibited the response at 10^{-7} M (Fig. 5B). The H3 receptor agonist, α -methylhistamine (10^{-6} M), significantly increased $[Ca^{2+}]_i$ and was 173.1 ± 33.9 nM. Similar to the H2 agonist, the H3 agonist stimulation was significantly blocked by AG1478 10^{-8} M to 32.8 ± 13.7 nm and completely

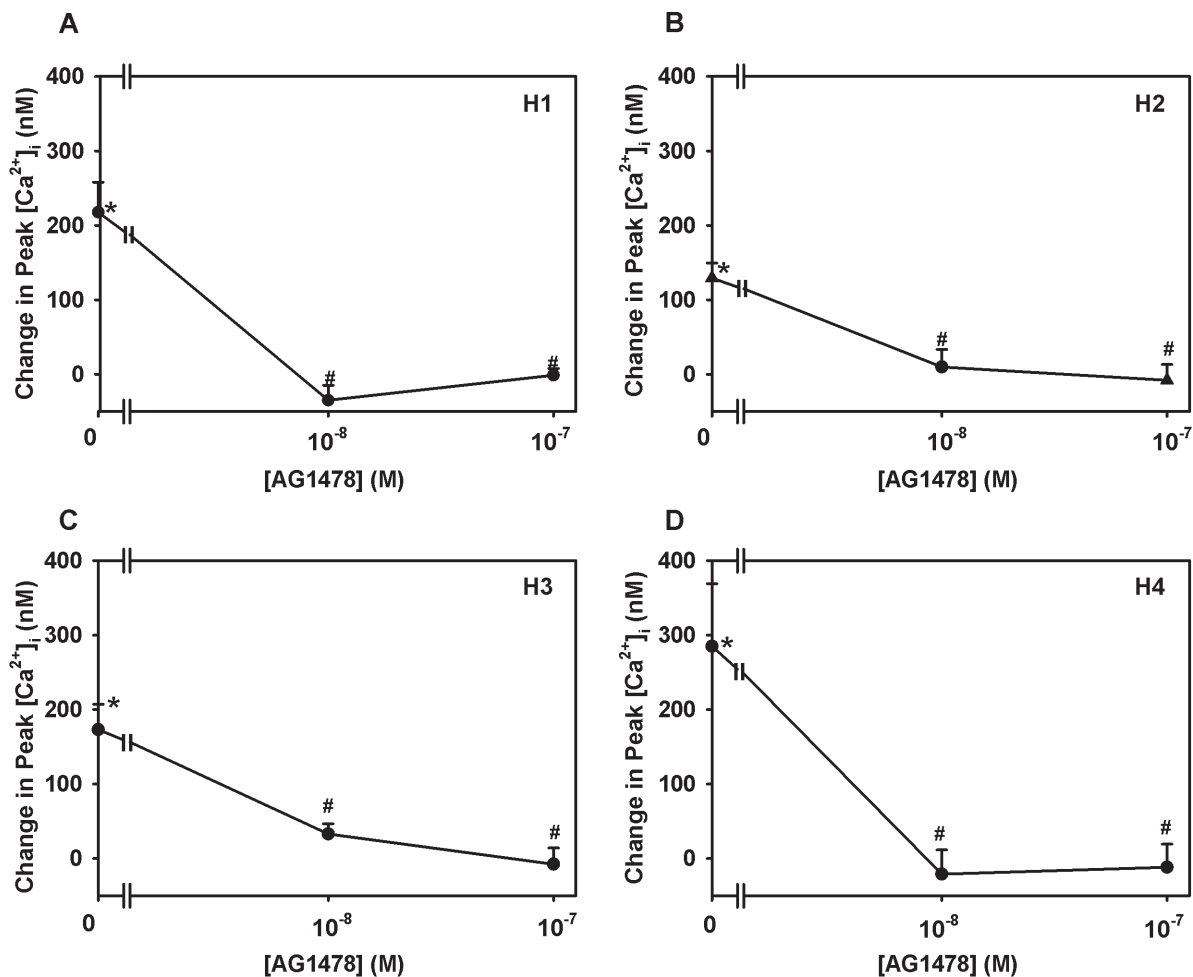


FIGURE 5. Histamine receptor subtype-stimulated increase in $[Ca^{2+}]_i$ is blocked by inhibition of the EGFR. Cultured rat goblet cells were preincubated with AG1478 (10^{-8} and 10^{-7} M) for 30 minutes prior to stimulation with agonists specific to each receptor subtype and increase in peak $[Ca^{2+}]_i$ was measured. The H1 receptor agonist histamine dimaleate (10^{-6} M) is shown in A. The H2 receptor agonist, anthamine (10^{-4} M), is shown in B. The H3 receptor agonist, α -methylhistamine (10^{-6} M), is shown in C, while the H4 receptor agonist, 4-methylhistamine (10^{-5} M), is shown in D. Data are mean \pm SEM from three independent experiments. Asterisk indicates significant difference from basal value. Pound sign indicates significant difference from agonist alone.

inhibited the response at 10^{-7} M (Fig. 5C). Similar to the other receptors, activation of the H4 receptor with methylhistamine (10^{-5} M) increased $[Ca^{2+}]_i$ and was 285.0 ± 84.3 nM. This response was completely inhibited by AG1478 at both concentrations. These data indicate that activation of the EGFR leads to increase in $[Ca^{2+}]_i$ stimulated by the four histamine receptor subtypes.

Inhibition of ADAM17 MMP Blocks Increase in $[Ca^{2+}]_i$ Stimulated by Histamine and Specific Agonists for Each Histamine Receptor Subtype in Conjunctival Goblet Cells

Activation of the EGFR occurs via ectodomain shedding when an MMP cleaves a membrane-bound member of the EGF family. The released growth factor binds to and activates the EGFR. To determine if ectodomain shedding occurs in conjunctival goblet cells and leads to activation of the histamine receptors, cells were incubated with TAPI-1 (10^{-6} M) for 30 minutes, an inhibitor of the MMP, ADAM17/TACE. $[Ca^{2+}]_i$ was measured in response to histamine and the specific agonists for each

receptor subtype. As shown in Figure 6, histamine significantly increased $[Ca^{2+}]_i$ to 252.8 ± 37.6 nM. This was significantly inhibited by TAPI-1 and was 56.5 ± 31.8 nM. In the same cells, each agonist specific receptor also significantly increased $[Ca^{2+}]_i$, and each was inhibited by TAPI-1 (Fig. 6). In previous studies, the cholinergic agonist carbachol (Cch) transactivated the EGFR via ADAM17/TACE.¹⁵ Therefore, Cch was used as a positive control in this study. Cch-stimulated increase in $[Ca^{2+}]_i$ was significantly inhibited by TAPI-1 from 285.9 ± 66 to 62.5 ± 21.6 nM. Thus, all four histamine receptor subtypes utilize ADAM17 to transactivate the EGFR.

Inhibition of EGR Blocks Histamine-Stimulated Phosphorylation of AKT but Not ERK1/2 in Conjunctival Goblet Cells

Multiple signaling pathways are stimulated when a member of the EGF family binds to and activates EGFR. Two such pathways involve AKT and ERK1/2. To determine if either of these kinases were activated by histamine in conjunctival cells, Western blot analysis was performed with antibodies against

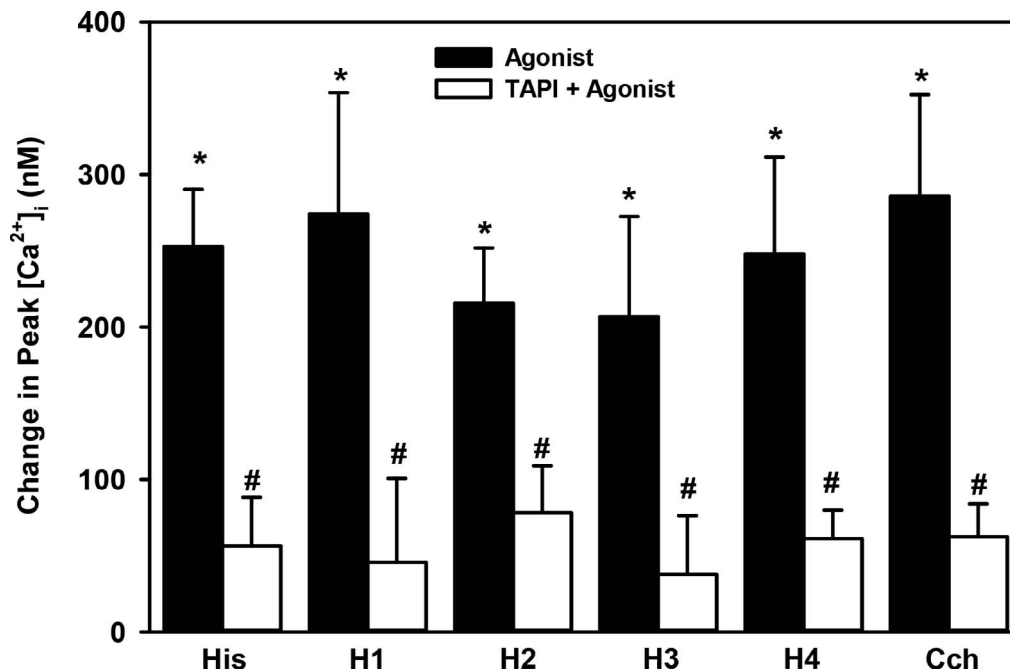


FIGURE 6. Histamine receptor subtype-stimulated increase in $[Ca^{2+}]_i$ is blocked by inhibition of MMP. Cultured rat goblet cells were preincubated with the MMP inhibitor TAPI-1 (10^{-6} M) for 30 minutes prior to stimulation with histamine, agonists specific to each receptor subtype, or carbachol (Cch) as a positive control. The increase in peak $[Ca^{2+}]_i$ was measured. The H1 receptor agonist was histamine dimaleate (10^{-6} M); H2 receptor agonist was amthamine (10^{-4} M); H3 receptor agonist was α -methylhistamine (10^{-6} M); and H4 receptor agonist was 4-methylhistamine (10^{-5} M). Data are mean \pm SEM from five independent experiments. *Asterisk* indicates significant difference from basal value. *Pound sign* indicates significant difference from agonist alone.

phosphorylated (active) AKT (pAKT) or ERK1/2 (pERK1/2) and standardized to total AKT or ERK1/2, respectively. Histamine (10^{-6} M) increased the activation of AKT 1.7 \pm 0.2-fold above basal, which was set to 1 (Fig. 7A). Preincubation with AG1478 10^{-7} and 10^{-6} M blocked phosphorylation of AKT to basal values. The pAKT value at AG1478 10^{-7} M was significantly decreased from histamine alone.

To determine if ERK1/2 was also inhibited by AG1478, Western blot analysis was performed for phosphorylated (active) and total ERK1/2 using the same samples as used for AKT. Histamine increased phosphorylation of ERK1/2 2.0 \pm 0.2-fold above basal (Fig. 7B). In cells preincubated with AG1478 10^{-7} and 10^{-6} M, there was no effect on this activity. Thus histamine transactivates the EGFR to activate AKT, but not ERK 1/2.

DISCUSSION

In conjunctival goblet cells, each of the histamine receptor subtypes transactivate the EGFR through activation of an MMP, most likely ADAM17. This leads to phosphorylation of AKT, but not ERK1/2, to increase $[Ca^{2+}]_i$ and stimulate mucin secretion (Fig. 8).

All four histamine receptor subtypes transactivate the EGFR. Other studies have demonstrated that the signaling pathways for each of the histamine receptors are similar but do have some important differences. The H1 receptor increases $[Ca^{2+}]_i$ through the $G\alpha_q$ family of G proteins and activation of phospholipase C (PLC) and release of inositol trisphosphate (IP3). The H2 receptor increases cAMP through activation of $G\alpha_s$. In addition, the H2 receptor activates $G\alpha_q$ followed by PLC/IP3 to increase $[Ca^{2+}]_i$. The H3 receptor is coupled to $G\alpha_i/o$ to decrease cAMP but increases $[Ca^{2+}]_i$ through activation of

PLC/IP3.^{27,28} The H4 receptor is also coupled to $G\alpha_i/o$ and also increases $[Ca^{2+}]_i$.²⁹ In cultured conjunctival goblet cells, all four receptor subtypes increase $[Ca^{2+}]_i$ and mucin secretion, confirming earlier studies.⁵ It is of interest that these increases are mediated through transactivation of the EGFR for all four subtypes, implying that blocking the EGFR alone could relieve histamine-mediated symptoms of allergic eye disease.

This study demonstrates that histamine activates multiple signaling pathways in conjunctival goblet cells. Previously, we demonstrated that histamine activates ERK1/2 and that inhibiting this activation blocked mucin secretion.⁷ In this study, histamine stimulated ERK phosphorylation. However, the phosphorylation of ERK1/2 was not inhibited by AG1478. This implies that histamine activates multiple signaling pathways, some of which are EGFR dependent (AKT activation) and some are EGFR independent (ERK1/2 activation).

Only the role of EGFR was addressed in this study and was shown to be transactivated. Using an antibody specific to phosphorylated EGFR, siRNA specific to the EGFR, and AG1478, which is highly selective for EGFR, these data demonstrated that histamine through all receptor subtypes transactivates the EGFR. The roles of the other members of the ErbB family were not investigated. As ErbB receptors form homo- and heterodimers, it is possible that the other members of family could form heterodimers with the EGFR to play a role in histamine-stimulated processes.

When stimulated by histamine, the peak phosphorylation of EGFR occurred after 30 minutes. In contrast, significant phosphorylation of the EGFR occurs after 5 minutes when stimulated with EGF. Previous studies have shown that conjunctival goblet cells stimulated with Cch transactivated the EGFR with a significant increase in activity after 10 minutes.³⁰ However, Hirota et al.¹⁹ found that histamine-

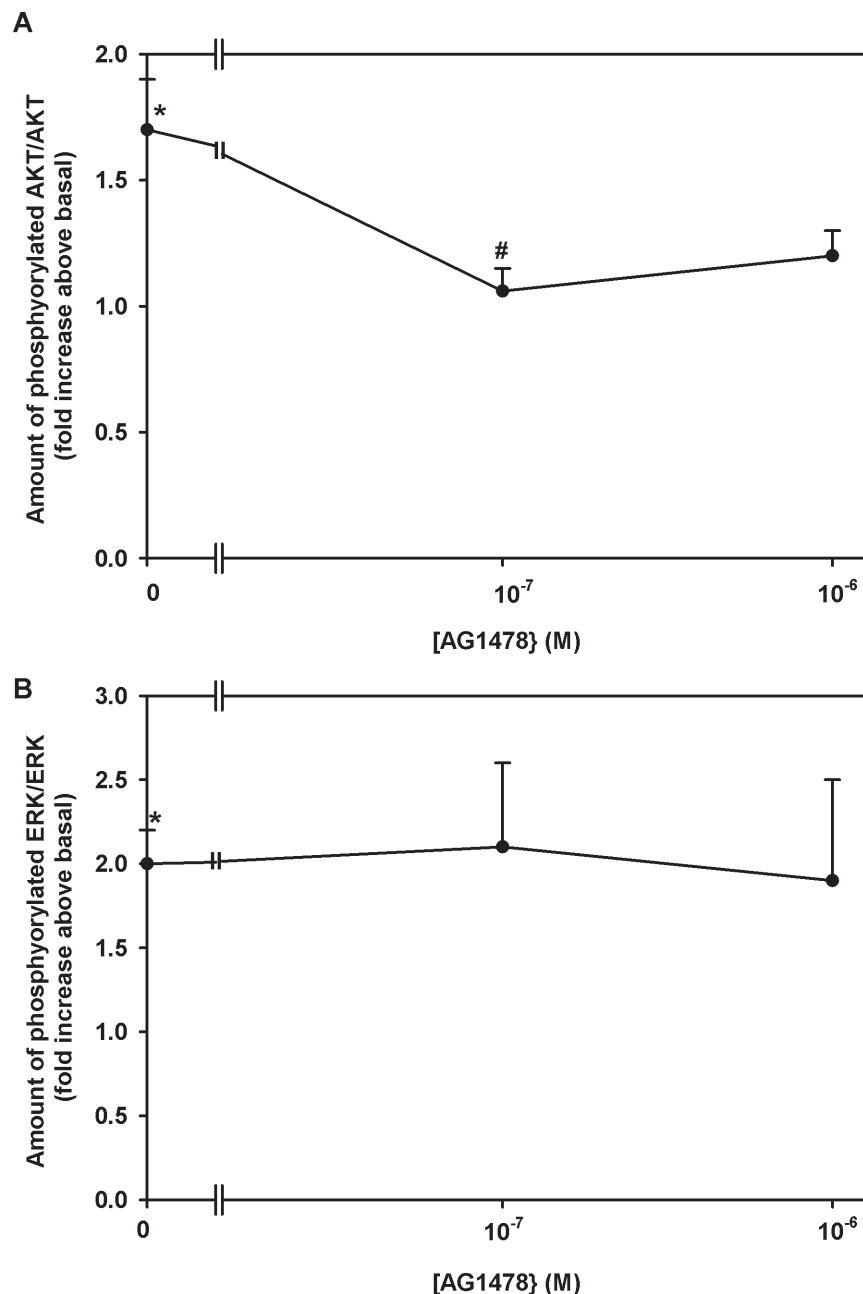


FIGURE 7. Histamine stimulation of AKT activity, but not ERK activity, was blocked by inhibition of the EGFR. Cultured rat conjunctival goblet cells were preincubated with AG1478 (10^{-8} and 10^{-7} M) for 30 minutes prior to stimulation with histamine (10^{-6} M) for 5 minutes, and the amount of phosphorylated (active) and total AKT (**A**) or phosphorylated and total ERK (**B**) was determined by Western blot analysis. Data are mean \pm SEM from three (**A**) or four (**B**) independent experiments. *Asterisk* indicates significant difference from basal value. *Pound sign* indicates significant difference from agonist alone.

increased phosphorylation of the EGFR peaked 2 hours after addition of histamine in bronchial epithelial cells. Longer activation times of the EGFR imply that the receptor stimulation occurs later in the signaling process. Consistent with this is that in goblet cells activation of the EGFR by histamine occurs after 30 minutes, allowing for ectodomain shedding to occur, compared to 5 minutes with exogenously added EGF, which binds directly to the EGFR.

All experiments in the present study were performed with conjunctival goblet cells from rats. Previous studies demon-

strated that human conjunctival goblet cells also contain all four histamine receptor subtypes. In addition, cultured human goblet cells respond to histamine to increase $[Ca^{2+}]_i$ and mucin secretion similar to results obtained with rats.^{5,10} Human and rat goblet cells also respond similarly when stimulated with EGF,²⁵ as well as cholinergic agonists,³⁰ resolvins, and leukotrienes.⁶ These data imply that rat conjunctival goblet cells are an excellent model for human goblet cells.

In conclusion, in conjunctival goblet cells, histamine, using all four receptor subtypes, transactivates the EGFR via an MMP,

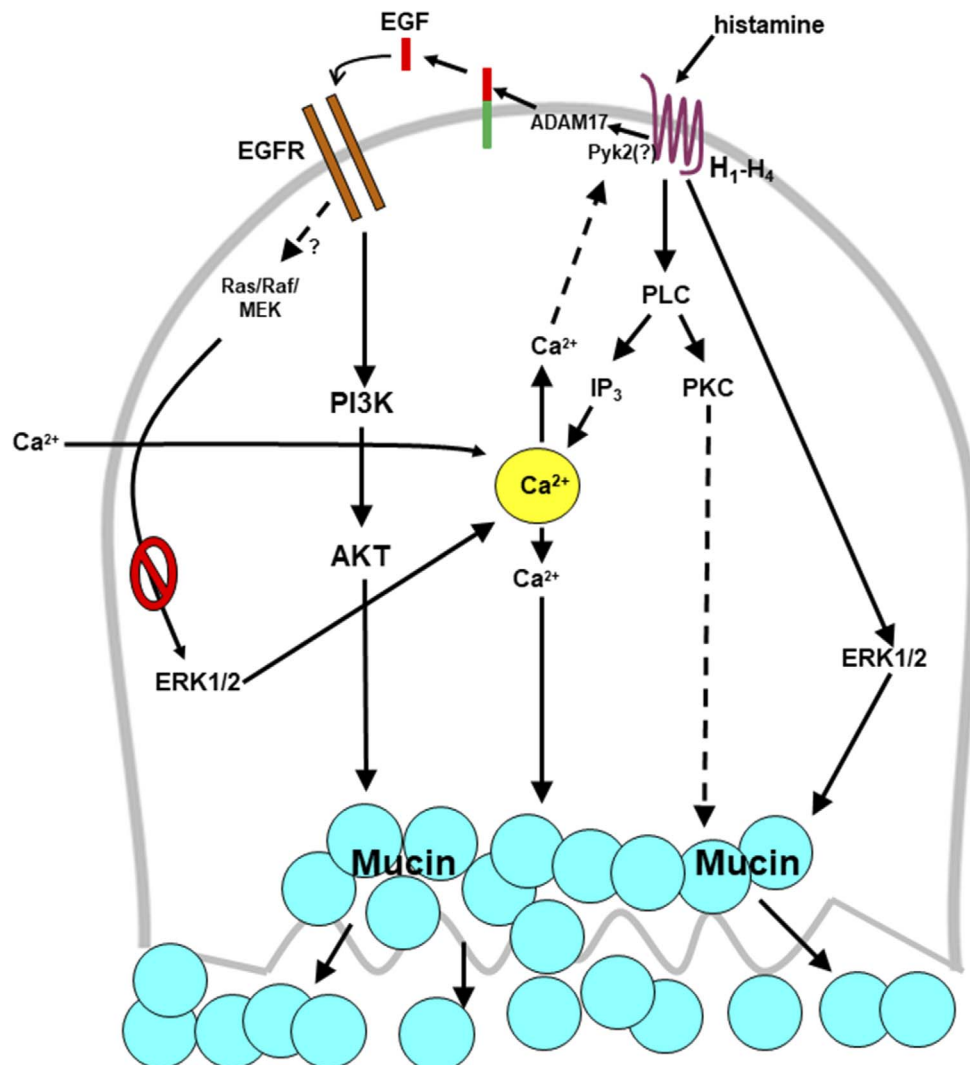


FIGURE 8. Schematic diagram of the signaling pathways activated by histamine. *Solid lines* indicate known pathways; *dotted lines* are hypothetical pathways. H, histamine receptors; EGFR also called ErbB1; Ras, small GTPase; Raf, serine/threonine kinase; MEK, mitogen-activated protein kinase; ERK1/2 also known as MAPK; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, serine/threonine kinase, also known as protein kinase B; PKC, protein kinase C.

likely ADAM17. This in turn phosphorylates AKT but not ERK1/2 to increase $[Ca^{2+}]_i$ and stimulate mucin secretion.

Acknowledgments

Supported by The Norwegian Research Council (ML) and the National Institutes of Health Grant EY019470 (DAD).

Disclosure: **M. He**, None; **M. Lippestad**, None; **D. Li**, None; **R.R. Hodges**, None; **T.P. Utheim**, None; **D.A. Dartt**, None

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