Cholinergic agonists transactivate EGFR and stimulate MAPK to induce goblet cell secretion

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Cholinergic agonists transactivate EGFR and stimulate MAPK to induce goblet cell secretion. Am J Physiol Cell Physiol 284: C988–C998, 2003. First published December 21, 2002; 10.1152/ajpcell.00582.2001.—Conjunctival goblet cells are the primary source of mucins in the mucous layer, the innermost layer of the tear film. Conjunctival goblet cell mucin secretion is under neural control because exogenous addition of parasympathetic agonists stimulates goblet cell secretion. To elucidate the intracellular signal pathways used by cholinergic agonists to stimulate goblet cell mucin secretion, we determined whether p42/p44 mitogen-activated protein kinase (MAPK) is activated during cholinergic agonist-stimulated mucin secretion. Rat conjunctiva was removed, preincubated with or without antagonists, and stimulated with the cholinergic agonist carbachol (10−4 M). Carbachol statistically significantly stimulated the phosphorylation of MAPK in a time- and concentration-dependent manner. U-0126, an inhibitor of MAPK activation, completely inhibited both the activation of MAPK and goblet cell secretion stimulated by carbachol. The M1 muscarinic agonist pirenzepine, the M2 muscarinic agonist gallamine, and the M3 muscarinic receptor antagonist N-(3-chloropropyl)-4-piperidinyl diphenylacetate (4-DAMP) also inhibited carbachol-stimulated MAPK activation. Increasing the intracellular Ca2+ concentration with a Ca2+ ionophore increased MAPK activation, and chelation of extracellular Ca2+ inhibited carbachol-stimulated activation. Carbachol also increased tyrosine phosphorylation of Pyk2, p60Src, and the epidermal growth factor receptor (EGFR). The Src inhibitor PP1 and the EGFR inhibitor AG-1478 completely inhibited carbachol-stimulated MAPK activation. AG-1478 also inhibited goblet cell secretion. We conclude that carbachol transactivates the EGFR to activate MAPK, leading to conjunctival goblet cell secretion. In addition, carbachol also activates Pyk2 and p60Src that could play a role in the transactivation of the EGFR.

THE CONJUNCTIVA is a mucin-secreting epithelium that lines the inner surface of the upper and lower eyelids, and proper function of this epithelium is crucial to clear vision. Conjunctival epithelium consists of layers of stratified squamous cells. The number of cell layers varies with location but is 5–12 layers thick with tight junctions between cells (15). Goblet cells are interspersed throughout the epithelium either as single cells or in clusters, depending on species (18, 30, 37), and extend through the cell layers to the basement membrane. Underlying the epithelium is a highly vascularized stroma containing nerves, blood vessels, and lymphoid tissue (15). Goblet cells are highly polarized epithelial cells present in tissues such as the trachea, colon, pancreatic ducts, and conjunctiva. The primary function of goblet cells is to synthesize, store, and secrete mucins (12). Conjunctival goblet cells are the primary source for mucins in the mucous layer of the tear film, which serves to protect the cornea and conjunctiva from bacterial infection and to facilitate the occurrence of a smooth refractive surface necessary for clear vision (33). Deterioration of the ocular surface occurs when the tear film is disrupted because of the loss of either goblet cells or their ability to secrete mucins. This can lead to vision impairment.

Similar to secretion from goblet cells in other tissues, conjunctival goblet cell mucin secretion is under neural control (17, 38, 39). We previously showed (6, 22) that parasympathetic and sympathetic nerves surround the basolateral membranes of goblet cells in rat conjunctiva and that stimulation of the afferent sensory nerves in the cornea triggers mucin secretion from goblet cells, probably through efferent parasympathetic or sympathetic nerves in the conjunctiva. In addition, we found (22, 35) that exogenous addition of carbachol, an analog of the parasympathetic neurotransmitter acetylcholine acting through the M2 and M3 muscarinic receptors, and vasoactive intestinal peptide (VIP) stimulate conjunctival goblet cell secretion.

Classically, two major pathways are involved when G protein-coupled receptor (GPCR) agonists such as carbachol are used. These two pathways involve the stimulation of protein kinase C (PKC) and mobilization of intracellular Ca2+. In most tissues, after cholinergic
agonists bind to their receptors, they activate phospholipase C to generate inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG) (2). IP$_3$ interacts with receptors on the endoplasmic reticulum to release intracellular Ca$^{2+}$, and DAG interacts with and activates PKC (3, 32). We showed (7) that Ca$^{2+}$ plays an important role in goblet cell mucin secretion in rat conjunctiva. Increasing intracellular Ca$^{2+}$ with a Ca$^{2+}$-ionophore stimulates mucin secretion from goblet cells, whereas chelation of extracellular Ca$^{2+}$ inhibits cholinergic agonist-induced mucin secretion. We also showed that several isoforms of PKC are present in conjunctival goblet cells and that activation of PKC with phorbol esters leads to goblet cell mucin secretion (7).

It is now well established that the mitogen-activated protein kinase (MAPK) pathway can also be activated by GPCR. There are at least three members of the MAPK family. They are the MAPKs [also known as extracellular signal-related kinases (ERKs) or p42/p44 MAPK], the p38-MAPKs, and the c-Jun NH$_2$-terminal kinases (JNKs) (40). Classically, the p42/p44 MAPK pathway, hereafter abbreviated as MAPK, is involved in the signaling pathway of growth factors such as epidermal growth factor (EGF) (36). This activation involves autophosphorylation of the EGF receptor (EGFR), which serves as a scaffold for the recruitment of the adaptor proteins Shc and Grb2. The Ras-GTPase activating protein (RasGAP), associated with Grb2, becomes active when it associates with the plasma membrane to activate Ras (24). The activation of Ras initiates the activation of a cascade of protein kinases, namely Raf (MAPK kinase kinase), MEK (MAPK kinase), and, ultimately, MAPK. There are several mechanisms by which GPCRs activate MAPK. One such mechanism involves the activation of the nonreceptor tyrosine kinase p60Src by G$_{8/3}$ subunits, which subsequently activates metalloproteinases causing the shedding of heparin-binding (HB)-EGF. HB-EGF then binds to and activates the EGFR (32a, 32b). Another mechanism involves activation of p60src by the focal adhesion kinase Pyk2, which is itself activated by Ca$^{2+}$ and PKC. The activated EGFR then serves as a scaffold for the Shc-Grb2 complex, leading to the activation of Ras and Raf (25). Increases in extracellular Ca$^{2+}$, as well as activation of PKC, were shown to activate MAPK (40). PKC can directly activate Raf, leading to activation of MAPK (28). Inhibition of phosphatidylinositol 3-kinase (PI3K) was also shown to inhibit MAPK activation, although the precise mechanism used by PI3K is unclear (40).

MAPK has been implicated in a variety of cellular processes such as cell proliferation and differentiation and gene expression (40). These effects are considered “long term,” with the time frame of these events being hours rather than minutes (40). Only a few studies have implicated MAPK in short-term effects with time courses such as we detect with for mucin secretion. In the current study, we show that MAPK is activated through transactivation of the EGFR during cholinergic stimulation of conjunctival goblet cells. This activation then leads to stimulation of mucin secretion.

**MATERIALS AND METHODS**

**Materials.** Antibodies that detect phosphorylated (active) p42/p44 MAPK, total p42 MAPK, EGFR, and total Pyk2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies that detect Pyk2 phosphorylated at tyrosine 881 and total p60src were from Biosource International (Camarillo, CA). An antibody to detect p60src phosphorylated on tyrosine 416 was obtained from Cell Signaling Technology (Beverly, MA). Antibodies against M$_1$, M$_2$, and M$_3$ muscarinic receptors were from Research & Diagnostic Antibodies (Bencic, CA). EGF was from Upstate Biotechnology (Lake Placid, NY). Pirenzipine, gallamine, and N-(3-chloropropyl)-4-piperidinyl diphenylacetate (4-DAMP) were from RBI. U-0126 and PP1 were from Biomol Research Labs (Plymouth Meeting, PA). Tyrphostin AG-1478 was from Calbiochem (San Diego, CA). Keratinocyte basal medium (KBM) was from Clonetics (San Diego, CA). Tris-glucose electrophoresis gel supplies were from Bio-Rad (Hercules, CA). Biotinylated *Ulex europeaus* agglutinin I (UEA-I) and the chemiluminescence reagents were from Pierce (Rockville, IL). All other reagents were from Sigma (St. Louis, MO).

**Methods.** All experiments conformed to the U.S. Department of Agriculture Animal Welfare Act (1985) and were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Male Sprague-Dawley rats (250–300 g) were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized for 1 min in CO$_2$ and decapitated, and both eyes were removed. The entire conjunctiva was dissected around the limbus of the cornea, placed on filter paper, and cut into two pieces (for a total of 4) for secretion experiments and four pieces (for a total of 8) for MAPK experiments. The pieces were precultured for 60 min in KBM at 37°C. 

*Immunoprecipitation and Western blotting.* After preculture, conjunctival pieces were incubated with varying concentrations of carbachol for 10 min or carbachol at 10$^{-4}$ M for varying times. We chose to use a 10-min incubation time with carbachol because preliminary experiments indicated that there was little difference in phosphorylation between 5 and 10 min (1.34- and 1.26-fold increase above basal, respectively). In some experiments, antagonists were added 10–30 min before the addition of carbachol. The pieces were removed from the medium and homogenized in ice-cold RIPA buffer (containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 µg/ml phenylmethylsulfonyl fluoride, 30 µg/ml aprotinin, and 1 mM sodium orthovanadate). The homogenate was centrifuged at 3,000 rpm for 30 min at 4°C, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose. Immunoreactive bands were visualized with the enhanced chemiluminescence method. The films were scanned with BDS Image and analyzed with NIH Image software.

For immunoprecipitation experiments, the supernatant was incubated overnight at 4°C in the presence of an anti-EGFR antibody. The immunoprecipitate was collected after addition of protein A agarose and centrifugation. The immune complex was washed three times with RIPA buffer containing 0.1% SDS and 1% Triton X-100 in 10 mM Tris-HCl, pH 8.0, and boiled for 5 min (23a). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with either anti-phosphotyrosine or anti-EGFR antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence method. The films were scanned with BDS Image and analyzed with NIH Image software.

*Measurement of goblet cell secretion.* Both eyes were removed, and the inferior conjunctiva was cut into four pieces.
as described previously (35). Conjunctival pieces were stimulated with the cholinergic agonist carbachol for 60 min in KBM at 37°C. We previously showed (35) that mucin secretion was linear for 10–120 min. Where indicated, the conjunctival pieces were preincubated with antagonists for 10 min before stimulation with carbachol. After stimulation, medium was collected and the tissue was homogenized in 0.1 M bicarbonate buffer containing 1% SDS. The amount of glycoconjugate, our marker of mucin secretion, was measured in both the medium and tissue by enzyme-linked lectin assay (ELLA) with the biotinylated lectin UEA-I as described previously (35).

Goblet cell culture. Goblet cells were grown in organ culture as described previously (25). In brief, the nictitating membranes and fornix were removed from rat conjunctiva, minced, and placed in culture with RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, and 100 μg/ml penicillin-streptomycin. After nodules of cells were observed, the tissue plug was removed and goblet cells were allowed to grow from the nodules.

Data presentation and statistical analysis. To determine whether MAPK was activated under the conditions tested, the amount of phosphorylated MAPK was determined by Western blot analysis with an anti-phosphorylated p42/p44 MAPK antibody. These values for both bands were summed and then normalized to the amount of total MAPK, as determined by Western blot analysis with an anti-p44 antibody. To determine whether Pyk2 and p60Src were activated, the amount of phosphorylated protein was determined by Western blot analysis with an antibody specific to the phosphorylated protein. The values were normalized to the amount of total protein, as determined by Western blot analysis with an antibody to total protein. Data for MAPK, Pyk2, and p60Src experiments were expressed as fold increase above control value, which was normalized to 1. For secretion experiments, data were expressed as a percentage of the amount of secreted glycoconjugate (amount in medium) to the total glycoconjugate (amount in medium + amount in tissue). For immunoprecipitation experiments, Western blot analysis was performed by using the immunoprecipitating antibody to normalize for the number of cells in each condition.

Data are expressed as means ± SE and were analyzed by least-squares regression or Student's t-test for paired data. p values < 0.05 were considered to represent statistically significant differences.

RESULTS

Cholinergic agonists stimulated MAPK activity in a concentration-dependent manner. Conjunctival pieces were incubated with increasing concentrations (10^{-5} – 10^{-3} M) of the cholinergic agonist carbachol for 10 min, and activation of MAPK was measured. EGF (10^{-7} M) was used as a positive control. A representative blot is shown in Fig. 1A. When the results from 12 independent experiments were quantified, carbachol, at all concentrations, statistically significantly increased MAPK activity (Fig. 1B). A maximum activation of 2.1 ± 0.4-fold increase over basal level was detected at a carbachol concentration of 10^{-4} M (n = 12). This is comparable to the amount of MAPK activity obtained in the presence of a maximum concentration of EGF (10^{-7} M), which significantly increased MAPK activity 1.8 ± 0.2-fold over basal activity (n = 4, Fig. 1B).

Inhibition of MAPK blocks conjunctival goblet cell mucus secretion. To determine whether activation of MAPK is involved in conjunctival goblet cell secretion, we tested the effects of the MEK inhibitor U-0126 on MAPK activation and goblet cell secretion (10). Conjunctival pieces were preincubated for 10 min with U-0126 (10^{-7} – 10^{-5} M) and stimulated with carbachol (10^{-4} M) for 10 min. Figure 2A shows that U-0126 at 10^{-6} and 10^{-5} M completely blocked carbachol-stimulated MAPK phosphorylation (n = 4). U-0126, at any concentration tested, had no effect on basal MAPK activation. Similar concentrations of U-0126 have been used to inhibit MAPK activity in other cell types (26, 27).

To determine the effects of U-0126 on mucin secretion, conjunctival pieces were preincubated for 10 min with U-0126 (10^{-5} M) before stimulation with carbachol (10^{-4}) for 60 min. Goblet cell secretion was measured with the ELLA method described above. Carbachol increased secretion 2.3 ± 0.3-fold over basal level (Fig. 2B; n = 5). This stimulation was also completely
inhibited after preincubation with U-0126. U-0126 alone had no effect on basal secretion (Fig. 2B). These results indicate that activation of MAPK by cholinergic agonists stimulated secretion from conjunctival goblet cells.

**Carbachol activates muscarinic receptors to stimulate MAPK activity.** We previously showed by immunofluorescence that M₁, M₂, and M₃ receptors were present in the conjunctiva, with M₂ and M₃ receptors present on goblet cells (37a). To determine whether the effects of carbachol on MAPK are mediated through muscarinic receptors in the conjunctiva, conjunctival pieces were preincubated with the nonselective muscarinic antagonist atropine (10⁻⁵ M) for 10 min before stimulation with carbachol (10⁻⁴ M) for 10 min. Atropine statistically significantly decreased the amount of carbachol-stimulated MAPK activity by 87% (data not shown). It should be noted, however, that atropine alone had nonspecific effects and caused a significant increase in MAPK activity. Whereas these results suggest that cholinergic agonists used muscarinic receptors to stimulate conjunctival goblet cell secretion, we determined which subtypes of muscarinic receptors are involved in carbachol-stimulated MAPK. Conjunctival pieces were preincubated for 10 min with the selective M₁ antagonist pirenzepine (10⁻⁵ M) (13), the selective M₂ antagonist gallamine (10⁻⁵ M) (13), or the M₁/M₃ antagonist 4-DAMP (10⁻⁵ M) (9) before stimulation with carbachol (10⁻⁴ M). Figure 3A is a representative blot, which shows that pirenzepine, gallamine, and 4-DAMP inhibited carbachol-induced MAPK phosphorylation. When all experiments were quantified by densitometry, carbachol significantly increased MAPK ac-
tivity 1.5 ± 0.1-fold over basal activity (Fig. 3B). Carbachol-stimulated MAPK activity was completely inhibited by incubation of conjunctival pieces with pirenzepine, gallamine, and 4-DAMP (Fig. 3B; \( n = 4 \)). None of the inhibitors alone had a statistically significant effect on basal MAPK activity. We conclude that cholinergic agonists activate M1, M2, and M3 receptors to activate MAPK in the conjunctiva.

Role of Ca\(^{2+}\) in MAPK activation. To investigate the role of Ca\(^{2+}\) in activation of MAPK, conjunctival pieces were incubated for 10 min with the Ca\(^{2+}\) ionophore ionomycin (\(10^{-7} - 10^{-5}\) M). Figure 4A is a representative blot showing that ionomycin increased phosphorylation of MAPK. When four independent experiments were quantified by densitometry, ionomycin statistically significantly increased MAPK activation to a 3.0 ± 0.5- and 4.2 ± 0.9-fold increase above basal level at 10\(^{-7}\) M and 10\(^{-6}\) M, respectively (\( n = 6 \); Fig. 4B).

When extracellular Ca\(^{2+}\) was chelated by preincubating conjunctival pieces with 2 mM EGTA for 60 min before stimulation with carbachol (10\(^{-4}\) M), phosphorylation of MAPK was decreased (Fig. 4C). When four independent experiments were analyzed, chelation of Ca\(^{2+}\) decreased the amount of carbachol-induced MAPK phosphorylation 61% from 1.5 ± 0.2 to 1.2 ± 0.3 (Fig. 4D). These results indicate that although an increase in intracellular Ca\(^{2+}\) can activate MAPK, the cholinergic agonist response is only partially dependent on Ca\(^{2+}\).

Time dependence of carbachol-stimulated Pyk2, p60Src, and MAPK phosphorylation. Because Ca\(^{2+}\) is known to activate Pyk2 in other tissues, we determined whether carbachol activates Pyk2 in rat conjunctiva in a time-dependent manner. Conjunctival pieces were incubated with carbachol (10\(^{-4}\) M) for 0–30 min. The pieces were homogenized, and the amount of phosphorylated and total Pyk2 was determined by Western blot analysis. As shown in Fig. 5A, carbachol statistically significantly increased phosphorylation of Pyk2 at 30s and 1 and 5 min of stimulation to 2.0 ± 0.3-, 2.6 ± 0.2-, and 2.1 ± 0.3-fold increase over basal level, respectively (\( n = 4 \)). The amount of activated Pyk2 remained elevated for at least 30 min.

Because Pyk2 can activate p60Src, we determined whether carbachol activates p60Src. The same samples analyzed for Pyk2 were analyzed for p60Src activation. Phosphorylated and total p60Src was determined by Western blot analysis. As shown in Fig. 5B, carbachol statistically significantly increased phosphorylation of p60Src at 1 and 5 min of stimulation to 1.8 ± 0.2- and 3.0 ± 0.5-fold increase over basal level, respectively, before returning to basal levels by 30 min (\( n = 4 \)).

These same samples were then analyzed for MAPK activation. As shown in Fig. 5C, carbachol stimulated...
phosphorylation of MAPK after 5 and 10 min. MAPK phosphorylation was statistically significantly increased to 1.8 ± 0.1-fold over basal level after 5 min of stimulation and to 1.3 ± 0.0-fold after 10 min. MAPK phosphorylation decreased to 1.1 ± 0.3-fold increase over basal level after 30 min (Fig. 5C; n = 3).

We previously showed (35) that the time course for mucin secretion is linear from 10 (the earliest time point we were able to measure) to 120 min. These results indicate that Pyk2 is first activated by cholinergic agonist stimulation, followed by p60Src phosphorylation and, finally, MAPK activation.

**Effect of inhibition of p60Src on carbachol-stimulated MAPK activation.** To determine whether activation of p60Src is involved in carbachol-stimulated MAPK activation, conjunctival pieces were preincubated for 30 min with the p60Src inhibitor PP1 (10⁻⁵ M) (30) before stimulation with carbachol (10⁻⁴ M) for 10 min. The pieces were homogenized, and the amount of phosphorylated and total p60Src or MAPK was determined by Western blot analysis. Figure 6A is a representative blot showing that PP1 inhibited the carbachol-stimulated phosphorylation of p60Src. Figure 6B is a representative blot showing that PP1 inhibited the carbachol-stimulated phosphorylation of MAPK. When three independent experiments were analyzed, carbachol significantly increased MAPK activation 1.6 ± 0.1-fold over basal level (Fig. 6C). Preincubation with PP1 completely inhibited the carbachol-stimulated increase in MAPK activity. PP1 alone did not have a significant effect on basal MAPK activation. These results indicate that PP1 inhibits activation of p60Src in the conjunctiva and that p60Src plays a role in cholinergic agonist-stimulated MAPK activation.

**Carbachol transactivates EGFR.** One possible mechanism by which cholinergic agonists activate MAPK involves transactivation of the EGFR. To determine whether carbachol transactivates the EGFR in the conjunctiva, pieces were stimulated with either carbachol (10⁻⁴ M) for 10 min or EGF (10⁻⁷ M) for 5 min as a positive control. The pieces were then homogenized in RIPA buffer, and immunoprecipitation experiments were carried out with an anti-EGFR antibody. The proteins were analyzed by Western blotting with an anti-phosphotyrosine antibody to determine whether the EGFR was activated. To correct for the amount of tissue in each sample, Western blot analysis was also performed with the anti-EGFR antibody. As shown in Fig. 7, carbachol increased the tyrosine phosphorylation of the EGFR 1.7 ± 0.2-fold over basal level (n = 3). In comparison, EGF increased the tyrosine phosphorylation of the EGFR 3.2 ± 0.9-fold over basal level (n = 3). These results indicate that cholinergic agonists transactivate the EGFR in conjunctival pieces.

**Carbachol transactivates EGFR to activate MAPK and stimulate goblet cell secretion.** To determine whether transactivation of the EGFR plays a role in MAPK activation, conjunctival pieces were preincubated for 10 min with an inhibitor of the tyrosine kinase activity of the EGFR, trypthostin AG-1478 (10⁻⁸–10⁻⁶ M) (8), before stimulation with carbachol (10⁻⁴ M) for 10 min. Although 10⁻⁸ M AG-1478 did not inhibit carbachol-stimulated MAPK activation, it was inhibited 97% and 87% at 10⁻⁷ and 10⁻⁶ M AG-1478,
respectively (Fig. 8A). AG-1478, at any concentration tested, did not have a statistically significant effect on the basal MAPK activity (Fig. 8A). Similar concentrations of AG-1478 have been used in other cell types to inhibit the EGFR (8, 35a).

We next determined whether transactivation of the EGFR by carbachol plays a role in goblet cell secretion. Conjunctival pieces were preincubated for 10 min with AG-1478 (10^{-7} M) and stimulated with carbachol (10^{-4} M) for 60 min, and secretion was measured with ELLA. Carbachol statistically significantly increased secretion to 2.2 ± 0.6-fold over basal level, whereas AG-1478 completely inhibited this increase (Fig. 8B). AG-1478 alone did not have an effect on basal secretion. These results indicate that the cholinergic agonist carbachol transactivates the EGFR to activate MAPK and stimulate goblet cell secretion.

EGF and cholinergic agonists stimulated MAPK activity in a concentration- and time-dependent manner in cultured rat goblet cells. Because the conjunctiva is composed of several cell types, we determined whether MAPK is present in goblet cells by using a recently developed primary culture (37a). In these purified rat goblet cells, EGF stimulated MAPK in a concentration-dependent manner, with a maximum MAPK activation at a concentration of 10^{-7} M (Fig. 9A). We determined whether carbachol stimulated MAPK activation. As shown in Fig. 9B, carbachol stimulated MAPK activation in a concentration-dependent manner, with a maximum MAPK activation at a concentration of 10^{-4} M. Because all three muscarinic receptor antagonists inhibited the cholinergic agonist-induced increase in MAPK activity in the conjunctiva, we determined whether goblet cells contained the three muscarinic receptor subtypes by using antibodies specific to M1, M2, and M3 muscarinic receptors. Western blot analysis of cultured goblet cells demonstrated that M1, M2, and M3 muscarinic receptors were present in these cells (Fig. 9C). These results indicate that three types of muscarinic receptors and MAPK are present and can be activated by EGF and cholinergic agonists in cultured goblet cells.

Fig. 6. Effect of PP1 on CCh-induced MAPK activation. Rat conjunctival pieces were preincubated with PP1 (10^{-5} M) for 30 min before stimulation with CCh (10^{-4} M) for 10 min. Pieces were homogenized, and proteins were separated by SDS-PAGE. Western blot analysis was performed with an antibody to phosphorylated p60Src and total p60Src (A) or phosphorylated p42/p44 MAPK and total p42 MAPK (B). Representative blots are shown. Densitometric analyses were performed, and the data shown in C represent means ± SE (n = 3) of phosphorylated MAPK. *Statistical significance compared with basal secretion; †statistical significance compared with CCh alone.

Fig. 7. Effect of CCh on the activation of EGFR. Rat conjunctival pieces were incubated with CCh (10^{-4} M) for 10 min or with EGF (10^{-7} M) for 5 min as a positive control. The pieces were homogenized, and the EGFR was immunoprecipitated (IP). Western blot analysis was performed with an anti-phosphotyrosine (pTyr) antibody. Amount of EGFR added was standardized by reblotting with anti-EGFR antibody. A representative blot is shown in A. Data in B represent means ± SE from 3 independent experiments.
DISCUSSION

In the present study we have shown that cholinergic agonists transactivate the EGFR, which in turn activates MAPK to lead to mucin secretion. Thus MAPK has a direct role in stimulating mucin secretion from the goblet cells of the conjunctiva. In most tissues studied to date, MAPK has been implicated in long-term cellular effects such as gene transcription and cell proliferation and differentiation. There are, however, several studies in which it has been shown that MAPK plays a role, both positive and negative, in short-term effects such as ion and protein secretion. Two studies have shown that phorbol ester-induced peptide secretion from a neuroendocrine cell line is dependent on MAPK, as is the early phase of bombesin-induced cholecystokinin secretion from STC-1 cells (16, 31).

Fig. 8. Effect of AG-1478 on CCh-induced MAPK activation and goblet cell mucin secretion. Rat conjunctival pieces were preincubated with the EGFR inhibitor AG-1478 (10⁻⁸–10⁻⁶ M) for 10 min and then stimulated with CCh (10⁻⁴ M) for 10 min. The pieces were homogenized, and proteins were separated by SDS-PAGE. Western blot analysis was performed with an antibody to phosphorylated p42/p44 MAPK and total p42 MAPK. A: representative blot of 5 experiments is shown in inset. Densitometric analyses were performed, and the data shown represent means ± SE from 5 independent experiments. B: Conjunctival pieces were preincubated with the EGFR inhibitor AG-1478 (10⁻⁷ M) for 10 min and then stimulated with CCh (10⁻⁴ M) for 1 h. The pieces were removed and homogenized, and the amount of mucin secreted into the supernatant and the amount of mucin remaining in the tissue were measured by ELLA. Data represent means ± SE from 5 independent experiments. *Statistical significance compared with basal secretion; †statistical significance compared with CCh alone.

Fig. 9. Effect of EGF and carbachol on MAPK activation in cultured rat goblet cells. A: confluent cultures of goblet cells from rat conjunctiva were incubated with EGF (10⁻⁶–10⁻⁷ M) for 10 min. The cells were homogenized, and proteins were separated by SDS-PAGE. Western blot analysis was performed with an antibody to phosphorylated p42/p44 MAPK and total p42 MAPK. A: representative blot of 5 experiments is shown in inset. Densitometric analyses were performed, and the data shown represent means ± SE from 5 experiments. B: confluent cultures of goblet cells from rat conjunctiva were incubated with CCh (10⁻⁵–10⁻³ M) for 10 min. The cells were homogenized, and proteins were separated by SDS-PAGE. Western blot analysis was performed with an antibody to phosphorylated p42/p44 MAPK and total p42 MAPK. Densitometric analyses were performed, and the data represent means of 4 independent experiments. *Significant difference from basal level. C: confluent cultures of goblet cells (G) and rat conjunctiva (C) were homogenized, and proteins were separated by SDS-PAGE. Western blot analysis was performed with antibodies to M₁, M₂, and M₃ muscarinic receptors.
ever, although the conjunctival goblet cells require the transactivation of the EGFR, neither of these cases do. Keely et al. (20) showed that cholinergic agonists transactivate the EGFR, through intracellular Ca\(^{2+}\), Pyk2, and p60Src, to activate MAPK in T84 cells, similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study. In contrast to goblet cell secretion, activated MAPK in T84 cells inhibited cholinergic-agonist-stimulated Cl\(^-\) secretion, activated MAPK in T84 cells inhibited similar to the present study.

Activated MAPK is known to translocate to the nucleus on activation, where it phosphorylates transcription factors (4). It is thus involved in long-term cellular responses such as cell proliferation and differentiation. In the conjunctiva, the long-term effects of MAPK are not known. It is possible that MAPK plays a dual role in the conjunctiva, with the signal transduction pathway leading to both exocytosis (short-term effect) and activation of gene transcription (long-term effect).

The experiments in this study involved pieces of the entire conjunctiva. These pieces include not only goblet cells but also epithelial and stromal cells. Thus the MAPK activity measured in these experiments also includes MAPK activity from several different cell types. However, the biotinylated lectin used in the ELLA to measure goblet cell mucin secretion is specific to mucins present in rat goblet cells (35). Because inhibition of EGFR and MAPK with AG-1478 and U-0126, respectively, inhibited mucin secretion, EGFR must be present on goblet cells and transactivated by carbachol and MAPK must be activated in goblet cells.

We recently developed a primary culture of rat conjunctival goblet cells (37a). Western blot analysis showed that these cells express all three muscarinic receptors, M\(_1\), M\(_2\), and M\(_3\). We previously showed (35) that M\(_2\) and M\(_3\) receptors were present only on goblet cells in the conjunctiva, whereas M\(_1\) and M\(_2\) receptors were present on the stratified squamous cells. It is possible that M\(_1\) receptors are present on goblet cells in the conjunctiva and we were previously unable to detect them because of the difficulties inherent in the elastic nature of the conjunctiva. Nevertheless, MAPK activation was observed in cultured goblet cells when stimulated by both EGF and carbachol, with similar responses in magnitude seen in the cultured cells and the conjunctiva. MAPK activity was also completely inhibited by the use of specific inhibitors of the individual receptors, similar to what is observed from conjunctival pieces. We also found that AG-1478 inhibited carbachol-stimulated MAPK activation in the cultured cells similar to what is seen in goblet cells from the conjunctiva (unpublished data).

It is well established that cholinergic agonists generate two second messengers, IP\(_3\) and DAG. IP\(_3\) binds to receptors on the endoplasmic reticulum to release Ca\(^{2+}\) from intracellular stores, and DAG activates PKC (2). In the conjunctiva, we previously showed (35) that carbachol, through the M\(_2\) and M\(_3\) receptors (M\(_3\) > M\(_2\)), stimulated mucin secretion and that an increase in intracellular Ca\(^{2+}\) also stimulated mucin secretion. In this study, we showed that a carbachol-stimulated rise in intracellular Ca\(^{2+}\) leads to MAPK activation. One mechanism by which GPCR can activate MAPK is by transactivation of the EGFR. This can occur through the G\(_\alpha\gamma\) subunit stimulation of p60Src, which in turn activates metalloproteinases, which cleave HB-EGF (32a, 32b). The released HB-EGF can activate the EGFR either on the same cell or on neighboring cells. Transactivation of the EGFR can also occur through activation of Pyk2 by Ca\(^{2+}\) and PKC, which in turn activates p60Src, which then phosphorylates the EGFR (23b). In the rat conjunctiva, we have shown that Pyk2 is activated before p60Src and that p60Src is activated before MAPK. These data imply that this pathway (Ca\(^{2+}\), Pyk2, and p60Src) is involved in activation of MAPK to stimulate mucin secretion in conjunctival goblet cells.

The role of PKC in conjunctival goblet cell mucin secretion is less understood. Activation of PKC with the phorbol ester phorbol 12-myristate 13-acetate was shown to stimulate goblet cell mucin secretion (7). It is known in many tissues that PKC is involved in the regulation of Pyk2 (25). It was also shown that Ca\(^{2+}\) and PKC can activate Raf directly, bypassing the EGFR and thus activating MAPK (23b). However, the role that PKC plays in the activation of MAPK in this tissue is unknown and is the subject of further investigation.

Our results show that inhibition of M\(_1\), M\(_2\), and M\(_3\) muscarinic receptors with specific inhibitors each completely inhibits MAPK activation. We also observed similar results in cultured rat goblet cells (unpublished data). Evidence has emerged demonstrating that GPCRs are capable of forming homo- and heterodimers that can play a role in signal transduction (34). Indeed, many types of receptors have been shown to form such homodimers, including \(\alpha_{2}\) and \(\beta_{2}\)-adrenergic, opioid, and dopamine receptors. In addition, many types of GPCR form heterodimers between not only related family members but also receptors from other families (34). Such dimers can change the pharmacological and functional properties of the receptors. Chiacchio et al. (5) and Maggio et al. (27) showed that M\(_2\) and M\(_3\) muscarinic receptors are capable of forming heterodimers. Therefore, it is possible that the muscarinic receptors in the rat conjunctiva form heterodimers. If one of the receptors in the dimer is then inhibited, the activity of the dimer could also be diminished, leading to inhibition of MAPK activation.

It is of interest that M\(_1\), M\(_2\), and M\(_3\) muscarinic receptors appear to be linked to activation of MAPK in the rat conjunctiva. Classically, M\(_2\) muscarinic receptors are coupled to G\(_i\) and inhibition of adenylate cyclase, unlike M\(_1\) and M\(_3\) receptors, which are usually coupled to G\(_q\) to generate IP\(_3\) and DAG. Our results could be explained if the M\(_2\) receptors in the conjunctiva are also coupled to IP\(_3\) and DAG generation similar to the coupling of porcine M\(_2\) muscarinic receptors.
Another possibility is that G-coupled receptors can activate MAPK. Indeed, several studies have shown that this can occur (11, 14, 23, 26, 29). We cannot yet discriminate between these two hypotheses.

Activated MAPK is consistently seen under basal conditions because an intact tissue, probably containing growth factors, was used in these experiments. Thus it is possible that the addition of agonists would not cause a substantial further increase in MAPK activity or that the responses would be blunted. It is worth noting, however, that carbachol at 10^{-4} M gives a slightly larger response in MAPK activation than 10^{-5} M. This implies that cholinergic agonists are important activators of MAPK in the rat conjunctiva. Furthermore, cholinergic agonists activate MAPK and secretion to a similar extent (2.0-fold activation of MAPK and 2.2-fold, respectively). Thus activation of MAPK is a major mechanism that cholinergic agonists use to induce goblet cell secretion in the conjunctiva.

In conclusion, we have shown that in rat conjunctiva cholinergic agonists bind to muscarinic receptors, activate Fyk2 and p60Ssrc, and transactivate the EGFR to activate MAPK. Transactivation of the EGFR and activation of MAPK play a direct and important role in stimulating mucin secretion from the goblet cells of the conjunctiva.

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REFERENCES


